somewhat unselective transport across the capillary wall. Nor is the blood-brain barrier interrupted by continuous gaps or channels that run through the cells, thus allowing for unrestrained passage of drugs and other molecules.

Thus, a need exists for improved reagents and methods for enhancing delivery of compounds, including drugs, across epithelial tissues and endothelial tissues such as the skin and the blood-brain barrier. The present invention fulfills this and other needs.

SUMMARY OF THE INVENTION

The present invention provides methods for enhancing delivery of a compound into and across one or more layers of an animal epithelial or endothelial tissue. The methods involve contacting tissue with a conjugate that includes the compound and a delivery-enhancing transporter. The delivery-enhancing transporters, which are also provided by the invention, have sufficient guanidino or amidino moieties to increase delivery of the conjugate into and across one or more intact epithelial or endothelial tissue layers compared to delivery of the compound in the absence of the delivery-enhancing transporter. Typically, the delivery-enhancing transporters have from 6 to 25 guanidino or amidino moieties, and more preferably between 7 and 15 guanidino moieties.

The delivery-enhancing transporters and methods of the invention are useful for delivering drugs, diagnostic agents, and other compounds of interest across epithelial tissues such as the skin and mucous membranes. Delivery across the blood-brain barrier is also enhanced by the conjugates and methods of the invention. The methods and compositions of the invention can be used not only to deliver the compounds to the particular site of administration, but also provide systemic delivery.

In some embodiments, the delivery-enhancing transporter comprises 7-15 arginine residues or analogs of arginine. The delivery-enhancing transporter can have at least one arginine that is a D-arginine and in some embodiments, all arginines are D-arginine. The delivery-enhancing transporter can consist essentially of 5 to 50 amino acids, at least 50 percent of which are arginine. In some embodiments, at least 70% of the amino acids are arginines or arginine analogs. In some embodiments, the delivery-enhancing transporter comprises at least 5 contiguous arginines or arginine analogs.

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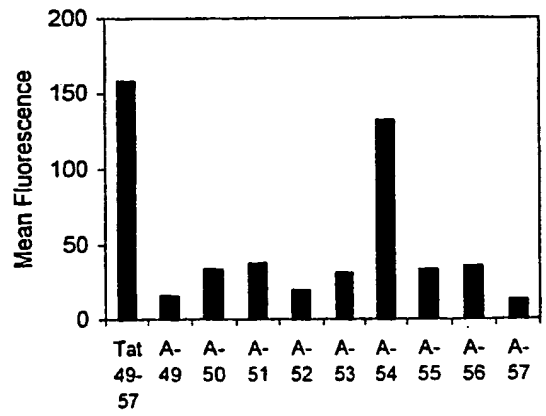
(54) Title: COMPOSITIONS AND METHODS FOR ENHANCING DRUG DELIVERY ACROSS AND INTO EPITHELIAL TISSUES



(57) Abstract: This invention provides compositions and methods for enhancing delivery of drugs and other agents across epithelial tissues, including the skin, gastrointestinal tract, pulmonary epithelium, and the like. The compositions and methods are also useful for delivery across endothelial tissues, including the blood brain barrier. The compositions and methods employ a delivery enhancing transport that has sufficient guanidino or amidino sidechain moieties to enhance delivery of a compound conjugated to the reagent across one or more layers of the tissue, compared to the non-conjugated compound. The delivery enhancing polymers include, for example, poly-arginine molecules that are preferably between about 6 and 25 residues in length.

WO 01/13957 A2

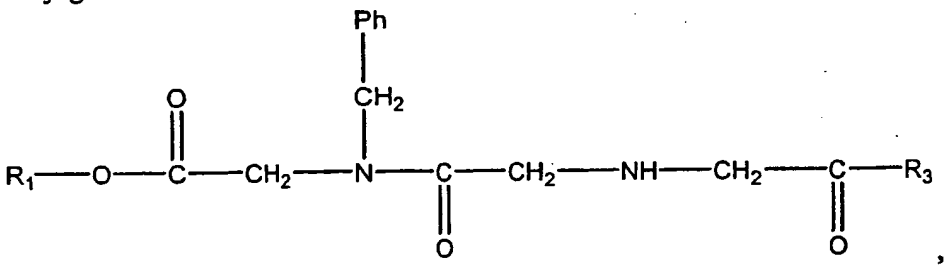
in particular, to drugs. The difficulties in delivering drugs across the skin result from the barrier property of skin. Skin is a structurally complex thick membrane that represents the body's border to the external hostile environment. The skin is composed of the epidermis, the dermis, the hypodermis, and the adenexal structures (epidermal appendages). The epidermis, the outermost epithelial tissue of the skin, is itself composed of several layers--the stratum corneum, the stratum granulosum, the stratum spinosum, and the stratum basale.

Compounds that move from the environment into and through intact skin must first penetrate the stratum corneum, the outermost layer of skin, which is compact and highly keratinized. The stratum corneum is composed of several layers of keratin-filled skin cells that are tightly bound together by a "glue" composed of cholesterol and fatty acids. The thickness of the stratum corneum varies depending upon body location. It is the presence of stratum corneum that results in the impermeability of the skin to pharmaceutical agents. The stratum corneum is formed naturally by cells migrating from the basal layer toward the skin surface where they are eventually sloughed off. As the cells progress toward the surface, they become progressively more dehydrated and keratinized. The penetration across the stratum corneum layer is generally the rate-limiting step of drug permeation across skin. *See, e.g.,* Flynn, G.L., In *Percutaneous Absorption: Mechanisms-Methodology-Drug Delivery, supra.* at pages 27-53.

After penetration through the stratum corneum layer, systemically acting drug molecules then must pass into and through the epidermis, the dermis, and finally through the capillary walls of the bloodstream. The epidermis, which lies under the stratum corneum, is composed of three layers. The outermost of these layers is the stratum granulosum, which lies adjacent to the stratum corneum, is composed of cells that are differentiated from basal cells and keratinocytes, which make up the underlying layers. having acquired additional keratin and a more flattened shape. The cells of this layer of the epidermis, which contain granules that are composed largely of the protein filaggrin. This protein is believed to bind to the keratin filaments to form the keratin complex. The cells also synthesize lipids that function as a "cement" to hold the cells together. The epidermis, in particular the stratum granulosum, contains enzymes such as aminopeptidases.

The next-outermost layer of the epidermis is the stratum spinosum, the principal cells of which are keratinocytes, which are derived from basal cells that comprise

delivery-enhancing transporter; R_4 is substituted or unsubstituted S, O, N or C; R_5 is OH, SH or NHR_6; R_6 is hydrogen, alkyl, aryl, acyl or allyl; k and m are each independently selected from 1 and 2; and n is 1 to 10. Preferably, X is selected from the group consisting of N, O, S, and CR_7R_8, wherein R_7 and R_8 are each independently selected from the group consisting of H and alkyl. In some embodiments, R_4 is S; R_5 is NHR_6; and R_6 is hydrogen, methyl, allyl, butyl or phenyl. In some embodiments, R_2 is benzyl; k, m, and n are each 1, and X is O. In some embodiments, the conjugate comprises structure **3** and R_2 is selected to obtain a conjugate half-life of between 5 minutes and 24 hours. In some embodiments, R_2 is selected to obtain a conjugate half-life of between 5 minutes and 24 hours. In some embodiments, the conjugate comprises structure **4**; R_4 is S; R_5 is NHR_6; and R_6 is hydrogen, methyl, allyl, butyl or phenyl. In some embodiments, the conjugate comprises structure **4**; R_5 is NHR_6; R_6 is hydrogen, methyl, allyl, butyl or phenyl; and k and m are each 1. One example of a conjugate is:



, where Ph is phenyl.

The invention also provides conjugates in which the release of the linker from the biological agent involves a first, rate-limiting intramolecular reaction, followed by a faster intramolecular reaction that results in release of the linker. The rate-limiting reaction can, by appropriate choice of substituents of the linker, be made to be stable at a pH that is higher or lower than physiological pH. However, once the conjugate has passed into and across one or more layers of an epithelial or endothelial tissue, the linker will be cleaved from the agent. An example of a compound that has this type of linker is structure **6**, as follows:

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a reaction scheme for the preparation of an α-chloroacetyl cyclosporin A derivative.

Figure 2 shows a general procedure for the coupling of cysteine-containing peptides to the α-chloro acetyl cyclosporin A derivative.

Figure 3 shows a reaction scheme for the coupling of the cyclosporin A derivative to a biotin-labeled peptide.

Figure 4 shows a reaction scheme for coupling of a cyclosporin A derivative to an unlabeled peptide.

Figure 5A-H show various types of cleavable linkers that can be used to link a delivery-enhancing transporter to a biologically active agent or other molecule of interest. Figure 5A shows an example of a disulfide linkage. Figure 5B shows a photocleavable linker which is cleaved upon exposure to electromagnetic radiation. Figure 5C shows a modified lysyl residue used as a cleavable linker. Figure 5D shows a conjugate in which the delivery-enhancing transporter T is linked to the 2'-oxygen of the anticancer agent, paclitaxel. The linking moiety includes (i) a nitrogen atom attached to the delivery-enhancing transporter, (ii) a phosphate monoester located para to the nitrogen atom, and (iii) a carboxymethyl group meta to the nitrogen atom, which is joined to the 2'-oxygen of paclitaxel by a carboxylate ester linkage. Figure 5E a linkage of a delivery-enhancing transporter to a biologically active agent, *e.g.*, paclitaxel, by an aminoalkyl carboxylic acid; a linker amino group is joined to a delivery-enhancing transporter by an amide linkage and to a paclitaxel moiety by an ester linkage. Figure 5F and G show chemical structures and conventional numbering of constituent backbone atoms for paclitaxel and "TAXOTERE™" (R' = H, R" = BOC). Figure 5G shows the general chemical structure and ring atom numbering for taxoid compounds.

Figure 6 displays a synthetic scheme for a chemical conjugate between a heptamer of L-arginine and cyclosporin A (panel A) and its pH dependent chemical release (panel B). The α-chloro ester (2) was treated with benzylamine in the presence of sodium iodide to effect substitution, giving the secondary amine (5). Amine (5) was treated with anhydride (6) and the resultant crude acid (7) was converted to its corresponding NHS ester (8). Ester (8) was then coupled with the amino terminus of hepta-L-arginine, giving the N-

Figure 17 shows a schematic diagram of a protocol for synthesizing a taxol 2'-chloroacetyl derivative.

Figure 18 shows a strategy by which a taxol 2'-chloroacetyl derivative is linked to an arginine heptamer delivery-enhancing transporter.

Figure 19 shows three additional taxol-r7 conjugates that can be made using the reaction conditions illustrated in Figure 18.

Figure 20 shows the results of a 3 day MTT cytotoxicity assay using taxol and two different linkers.

Figure 21: FACS cellular uptake assay of truncated analogs of Tat_{49-57} (Fl-ahx-RKKRRQRRR): Tat_{49-56} (Fl-ahx-RKKRRQRR), Tat_{49-55} (Fl-ahx-RKKRRQR), Tat_{50-57} (Fl-ahx-KKRRQRRR), and Tat_{51-57} (Fl-ahx-KRRQRRR). Jurkat cells were incubated with varying concentrations (12.5 μM shown) of peptides for 15 min at 23 °C.

Figure 22 shows FACS cellular uptake assay of alanine-substituted analogs of Tat_{49-57}: A-49 (Fl-ahx-AKKRRQRRR), A-50 (Fl-ahx-RAKRRQRRR), A-51 (Fl-ahx-RKARRQRRR), A-52 (Fl-ahx-RKKARQRRR), A-53 (Fl-ahx-RKKRAQRRR), A-54 (Fl-ahx-RKKRRARRR), A-55 (Fl-ahx-RKKRRQARR), A-56 (Fl-ahx-RKKRRQRAR), and A-57 (Fl-ahx-RKKRRQRRA). Jurkat cells were incubated with varying concentrations (12.5 μM shown) of peptides for 12 min at 23 °C.

Figure 23: FACS cellular uptake assay of *d*- and retro-isomers of Tat_{49-57}: *d*-Tat49-57 (Fl-ahx-rkkrrqrrr), Tat57-49 (Fl-ahx-RRRQRRKKR), and *d*-Tat57-49 (Fl-ahx-rrrqrrkkr). Jurkat cells were incubated with varying concentrations (12.5 μM shown) of peptides for 15 min at 23 °C.

Figure 24: FACS cellular uptake of a series of arginine oligomers and Tat_{49-57}: R5 (Fl-ahx-RRRRR), R6 (Fl-ahx-RRRRRR), R7 (Fl-ahx-RRRRRRR), R8 (Fl-ahx-RRRRRRRR), R9 (Fl-ahx-RRRRRRRRR), r5 (Fl-ahx-rrrrr), r6 (Fl-ahx-rrrrrr), r7 (Fl-ahx-rrrrrrr), r8 (Fl-ahx-rrrrrrrr), r9 (Fl-ahx-rrrrrrrrr). Jurkat cells were incubated with varying concentrations (12.5 μM shown) of peptides for 4 min at 23 °C.

Figure 25: Preparation of guanidine-substituted peptoids.

Figure 26: FACS cellular uptake of polyguanidine peptoids and *d*-arginine oligomers. Jurkat cells were incubated with varying concentrations (12.5 μM shown) of peptoids and peptides for 4 min at 23 °C.

The term "trans-epithelial" delivery or administration refers to the delivery or administration of agents by permeation through one or more layers of a body surface or tissue, such as intact skin or a mucous membrane, by topical administration. Thus, the term is intended to include both transdermal (*e.g.*, percutaneous adsorption) and transmucosal administration. Delivery can be to a deeper layer of the tissue, for example, and/or delivery to the bloodstream.

"Delivery enhancement, "penetration enhancement" or "permeation enhancement" as used herein relates to an increase in amount and/or rate of delivery of a compound that is delivered into and across one or more layers of an epithelial or endothelial tissue. An enhancement of delivery can be observed by measuring the rate and/or amount of the compound that passes through one or more layers of animal or human skin or other tissue. Delivery enhancement also can involve an increase in the depth into the tissue to which the compound is delivered, and/or the extent of delivery to one or more cell types of the epithelial or other tissue (*e.g.*, increased delivery to fibroblasts, immune cells, and endothelial cells of the skin or other tissue). Such measurements are readily obtained by, for example, using a diffusion cell apparatus as described in US Patent No. 5,891,462.

The amount or rate of delivery of an agent across and/or into skin or other epithelial or endothelial membrane is sometimes quantitated in terms of the amount of compound passing through a predetermined area of skin or other tissue, which is a defined area of intact unbroken living skin or mucosal tissue. That area will usually be in the range of about 5 cm^2 to about 100 cm^2, more usually in the range of about 10 cm^2 to about 100 cm^2, still more usually in the range of about 20 cm^2 to about 60 cm^2.

The terms "guanidyl," guanidinyl" and "guanidino" are used interchangeably to refer to a moiety having the formula $-HN=C(NH_2)NH$ (unprotonated form). As an example, arginine contains a guanidyl (guanidino) moiety, and is also referred to as 2-amino-5-guanidinovaleric acid or α-amino-δ-guanidinovaleric acid. "Guanidium" refers to the positively charged conjugate acid form. The term "guanidino moiety" includes, for example, guanidine, guanidinium, guanidine derivatives such as (RNHC(NH)NHR'), monosubstituted guanidines, monoguanides, biguanides, biguanide derivatives such as (RNHC(NH)NHC(NH)NHR'), and the like. In addition, the term "guanidino moiety" encompasses any one or more of a guanide alone or a combination of different guanides.

A "subunit," as used herein, is a monomeric unit that are joined to form a larger polymeric compound. The set of amino acids are an example of subunits. Each amino acid shares a common backbone (-C-C-N-), and the different amino acids differ in their sidechains. The backbone is repeated in a polypeptide. A subunit represents the shortest repeating pattern of elements in a polymer backbone. For example, two amino acids of a peptide are not considered a peptide because two amino acids would not have the shortest repeating pattern of elements in the polymer backbone.

The term "polymer" refers to a linear chain of two or more identical or non-identical subunits joined by covalent bonds. A peptide is an example of a polymer; peptides can be composed of identical or non-identical amino acid subunits that are joined by peptide linkages (amide bonds).

The term "peptide" as used herein refers to a compound made up of a single chain of D- or L- amino acids or a mixture of D- and L-amino acids joined by peptide bonds. Generally, peptides contain at least two amino acid residues and are less than about 50 amino acids in length. D-amino acids are represented herein by a lower-case one-letter amino acid symbol (*e.g.*, r for D-arginine), whereas L-amino acids are represented by an upper case one-letter amino acid symbol (*e.g.*, R for L-arginine). Homopolymer peptides are represented by a one-letter amino acid symbol followed by the number of consecutive occurrences of that amino acid in the peptide- (*e.g.*, R7 represents a heptamer that consists of L-arginine residues).

The term "protein" as used herein refers to a com-pound that is composed of linearly arranged amino acids linked by peptide bonds, but in contrast to peptides, has a well-defined conformation. Proteins, as opposed to peptides, generally consist of chains of 50 or more amino acids.

"Polypeptide" as used herein refers to a polymer of at least two amino acid residues and which contains one or more peptide bonds. "Polypeptide" encompasses peptides and proteins, regardless of whether the polypeptide has a well-defined conformation.

Description of the Preferred Embodiments

The present invention provides compositions and methods that enhance the transfer of compounds, including drugs and other biologically active compounds, into and

invention is increased more than 2-fold, still more preferably six-fold, still more preferably ten-fold, and still more preferably twenty-fold, over that obtained with tat residues 49-57.

Similarly, the delivery-enhancing transporters of the invention can provide increased delivery compared to a 16 amino acid peptide-cholesterol conjugate derived from the *Antennapedia* homeodomain that is rapidly internalized by cultured neurons (Brugidou *et al.* (1995) *Biochem. Biophys. Res. Commun.* 214: 685-93). This region, residues 43-58 at minimum, has the amino acid sequence RQIKIWFQNRRMKWKK. The *Herpes simplex* protein VP22, like tat and the *Antennapedia* domain, was previously known to enhance transport into cells, but was not known to enhance transport into and across endothelial and epithelial membranes (Elliot and O'Hare (1997) *Cell* 88: 223-33; Dilber *et al.* (1999) *Gene Ther.* 6: 12-21; Phelan *et al.* (1998) *Nature Biotechnol.* 16: 440-3). In presently preferred embodiments, the delivery-enhancing transporters provide significantly increased delivery compared to the *Antennapedia* homeodomain and to the VP22 protein.

Structure of Delivery-Enhancing Transporters

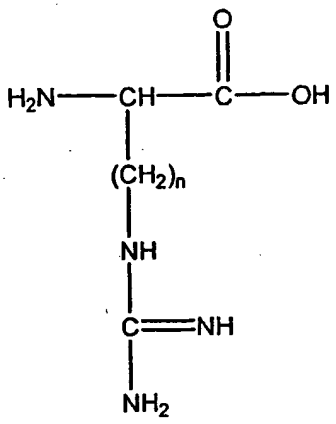
The delivery-enhancing transporters of the invention are molecules that have sufficient guanidino and/or amidino moieties to increase delivery of a compound to which the delivery-enhancing transporter is attached into and across one or more layers of an epithelial tissue (*e.g.*, skin or mucous membrane) or an endothelial tissue (*e.g.*, the blood-brain barrier). The delivery-enhancing transporters generally include a backbone structure to which is attached the guanidino and/or amidino sidechain moieties. In some embodiments, the backbone is a polymer that consists of subunits (*e.g.*, repeating monomer units), at least some of which subunits contain a guanidino or amidino moiety.

A. Guanidino and/or Amidino Moieties

The delivery-enhancing transporters typically display at least 5 guanidino and/or amidino moieties, and more preferably 7 or more such moieties. Preferably, the delivery-enhancing transporters have 25 or fewer guanidino and/or amidino moieties, and often have 15 or fewer of such moieties. In some embodiments, the delivery-enhancing transporter consists essentially of 50 or fewer subunits, and can consist essentially of 25 or fewer, 20 or fewer, or 15 or fewer subunits. The delivery-enhancing transporter can be as short as 5 subunits, in which case all subunits include a guanidino or amidino sidechain

More generally, it is preferred that each subunit contains a highly basic sidechain moiety which (i) has a pKa of greater than 11, more preferably 12.5 or greater, and (ii) contains, in its protonated state, at least two geminal amino groups (NH_2) which share a resonance-stabilized positive charge, which gives the moiety a bidentate character.

The guanidino or amidino moieties extend away from the backbone by virtue of being linked to the backbone by a sidechain linker. The sidechain atoms are preferably provided as methylene carbon atoms, although one or more other atoms such as oxygen, sulfur or nitrogen can also be present. For example, a linker that attaches a guanidino moiety to a backbone can be shown as:



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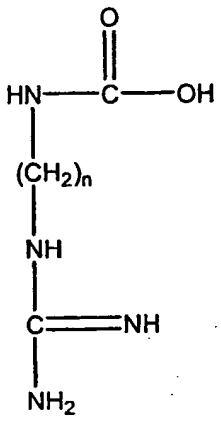
or



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In these formulae, n is preferably at least 2, and is preferably between 2 and 7. In some embodiments, n is 3 (arginine for structure 1). In other embodiments, n is between 4 and 6; most preferably n is 5 or 6. Although the sidechain in the exemplified formulae is shown as being attached to a peptide backbone (*i.e.*, a repeating amide to which the sidechain is attached to the carbon atom that is α to the carbonyl group, subunit 1) and a peptoid backbone (*i.e.*, a repeating amide to which the sidechain is attached to the nitrogen atom that is β to the carbonyl group, subunit 2), other non-peptide backbones are also suitable, as discussed in more detail herein. Thus, similar sidechain linkers can be attached to nonpeptide backbones (e.g., peptoid backbones).

In some embodiments, the delivery-enhancing transporters are composed of linked subunits, at least some of which include a guanidino and/or amidino moiety. Examples of suitable subunits having guanidino and/or amidino moieties are described below.

delivery-enhancing transporters used in the invention can also include variable spacing between sidechain moieties along the backbone.

A more detailed backbone list includes N-substituted amide (CONR replaces CONH linkages), esters (CO_2), keto-methylene ($COCH_2$) reduced or methyleneamino (CH_2NH), thioamide (CSNH), phosphinate (PO_2RCH_2), phosphonamidate and phosphonamidate ester (PO_2RNH), retropeptide (NHCO), trans-alkene (CR=CH), fluoroalkene (CF=CH), dimethylene (CH_2CH_2), thioether (CH_2S), hydroxyethylene ($CH(OH)CH_2$), methyleneoxy (CH_2O), tetrazole (CN_4), retrothioamide (NHCS), retroreduced ($NHCH_2$), sulfonamido (SO_2NH), methylenesulfonamido ($CHRSO_2NH$), retrosulfonamide ($NHSO_2$), and peptoids (N-substituted amides), and backbones with malonate and/or gem-diamino-alkyl subunits, for example, as reviewed by Fletcher *et al.* ((1998) *Chem. Rev.* 98:763) and detailed by references cited therein. Many of the foregoing substitutions result in approximately isosteric polymer backbones relative to backbones formed from α-amino acids.

As mentioned above, in a peptoid backbone, the sidechain is attached to the backbone nitrogen atoms rather than the carbon atoms. (*See e.g.*, Kessler (1993) *Angew. Chem. Int. Ed. Engl.* 32:543; Zuckerman et al. (1992) *Chemtracts-Macromol. Chem.* 4:80; and Simon et al. (1992) *Proc. Nat'l. Acad. Sci. USA* 89:9367.) An example of a suitable peptoid backbone is poly-(N-substituted)glycine (poly-NSG). Synthesis of peptoids is described in, for example, US Patent No. 5,877,278. As the term is used herein, transporters that have a peptoid backbone are considered "non-peptide" transporters, because the transporters are not composed of amino acids having naturally occurring sidechain locations. Non-peptoid backbones, including peptoid backbones, provide enhanced biological stability (for example, resistance to enzymatic degradation *in vivo*).

C. Synthesis of Delivery-enhancing Transporters

Delivery-enhancing transporters are constructed by any method known in the art. Exemplary peptide polymers can be produced synthetically, preferably using a peptide synthesizer (*e.g.*, an Applied Biosystems Model 433) or can be synthesized recombinantly by methods well known in the art. Recombinant synthesis is generally used when the delivery enhancing transporter is a peptide which is fused to a polypeptide or protein of interest.

In a third embodiment, the conjugate contains two agent moieties attached to each terminal end of the delivery-enhancing transporter. For this embodiment, it is presently preferred that the agent has a molecular weight of less than 10 kDa.

With regard to the first and third embodiments just mentioned, the agent is generally not attached to one any of the guanidino or amidino sidechains so that they are free to interact with the target membrane.

The conjugates of the invention can be prepared by straightforward synthetic schemes. Furthermore, the conjugate products are usually substantially homogeneous in length and composition, so that they provide greater consistency and reproducibility in their effects than heterogeneous mixtures.

According to an important aspect of the present invention, it has been found by the applicants that attachment of a single delivery-enhancing transporter to any of a variety of types of biologically active agents is sufficient to substantially enhance the rate of uptake of an agent into and across one or more layers of epithelial and endothelial tissues, even without requiring the presence of a large hydrophobic moiety in the conjugate. In fact, attaching a large hydrophobic moiety can significantly impede or prevent cross-layer transport due to adhesion of the hydrophobic moiety to the lipid bilayer of cells that make up the epithelial or endothelial tissue. Accordingly, the present invention includes conjugates that do not contain substantially hydrophobic moieties, such as lipid and fatty acid molecules.

Delivery-enhancing transporters of the invention can be attached covalently to biologically active agents by chemical or recombinant methods.

1. Chemical Linkages

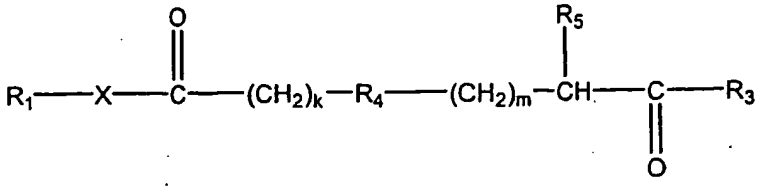
Biologically active agents such as small organic molecules and macromolecules can be linked to delivery-enhancing transporters of the invention via a number of methods known in the art (*see*, for example, Wong, S.S., Ed., *Chemistry of Protein Conjugation and Cross-Linking*, CRC Press, Inc., Boca Raton, FL (1991) , either directly (*e.g.*, with a carbodiimide) or via a linking moiety. In particular, carbamate, ester, thioether, disulfide, and hydrazone linkages are generally easy to form and suitable for most applications. Ester and disulfide linkages are preferred if the linkage is to be readily degraded in the cytosol, after transport of the substance across the cell membrane.

3. Releasable Linkers

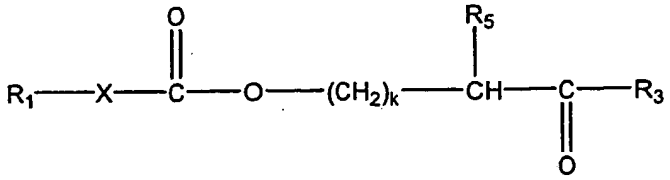
The biologically active agents are, in presently preferred embodiments, attached to the delivery-enhancing transporter using a linkage that is specifically cleavable or releasable. The use of such linkages is particularly important for biologically active agents that are inactive until the attached delivery-enhancing transporter is released. In some cases, such conjugates that consist of a drug molecule that is attached to a delivery-enhancing transporter can be referred to as prodrugs, in that the release of the delivery-enhancing transporter from the drug results in conversion of the drug from an inactive to an active form. As used herein, "cleaved" or "cleavage" of a conjugate or linker refers to release of a biological agent from a transporter molecule, thereby releasing an active biological agent. "Specifically cleavable" or "specifically releasable" refers to the linkage between the transporter and the agent being cleaved, rather than the transporter being degraded (*e.g.*, by proteolytic degradation).

In some embodiments, the linkage is a readily cleavable linkage, meaning that it is susceptible to cleavage under conditions found *in vivo*. Thus, upon passing into and through one or more layers of an epithelial and/or endothelial tissue, the agent is released from the delivery-enhancing transporter. Readily cleavable linkages can be, for example, linkages that are cleaved by an enzyme having a specific activity (*e.g.*, an esterase, protease, phosphatase, peptidase, and the like) or by hydrolysis. For this purpose, linkers containing carboxylic acid esters and disulfide bonds are sometimes preferred, where the former groups are hydrolyzed enzymatically or chemically, and the latter are severed by disulfide exchange, *e.g.*, in the presence of glutathione. The linkage can be selected so it is cleavable by an enzymatic activity that is known to be present in one or more layers of an epithelial or endothelial tissue. For example, the stratum granulosum of skin has a relatively high concentration of N-peptidase activity.

A specifically cleavable linker can be engineered onto a transporter molecule. For example, amino acids that constitute a protease recognition site, or other such specifically recognized enzymatic cleavage site, can be used to link the transporter to the agent. Alternatively, chemical or other types of linkers that are cleavable by, for example, exposure to light or other stimulus can be used to link the transporter to the agent of interest.



4



5

wherein:

R_1-X comprises the agent to be delivered;

X is a functional group on the agent, to which functional group the linker is attached;

Y is N or C;

R_2 is hydrogen, alkyl, aryl, acyl, or allyl;

R_3 comprises the delivery-enhancing transporter;

R_4 is substituted or unsubstituted S, O, N or C;

R_5 is OH, SH or NHR_6;

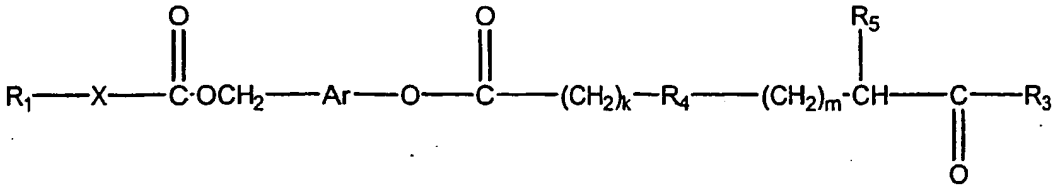
R_6 is hydrogen, alkyl, aryl, acyl or allyl;

k and m are each independently selected from 1 and 2; and

n is 1 to 10.

The agent to be delivered (*e.g.*, a drug or diagnostic agent) generally includes a functional group (designated as X in the formulae above) by which the linker is attached to the delivery-enhancing transporter. Examples of suitable functional groups for X include, for example, N, O, S, and CR_7R_8, wherein R_7 and R_8 are each independently selected from the group consisting of H and alkyl. If X is O, for example, release of the agent from the delivery-enhancing transporter and linker will yield the agent in its free alcohol form; if X is N, the free amine will result. Similarly, if X is S, release of the linker will yield the agent in the thiol form.

reaction occurs relatively quickly. An example of a conjugate having this type of linker is represented as structure **6**:



6

wherein:

R_1-X is the agent to be delivered;

X is a functional group on the agent, to which functional group the linker is attached;

Ar is an aryl group having the attached radicals arranged in an *ortho* or *para* configuration, which aryl group can be substituted or unsubstituted;

R_3 is the delivery-enhancing transporter;

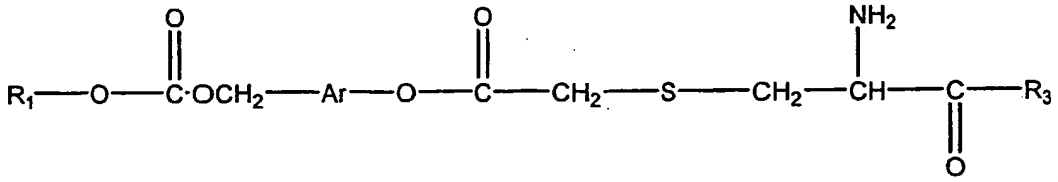
R_4 is substituted or unsubstituted S, O, N or C;

R_5 is OH, SH or NHR_6;

R_6 is hydrogen, alkyl, aryl, acyl or allyl; and

k and m are each independently selected from 1 and 2.

Examples of preferred conjugates of structure **6** include those in which R_4 is S, R_5 is NHR_6, and R_6 is hydrogen, methyl, allyl, butyl or phenyl. For example, a suitable conjugate of structure **6** is:



The self-immolating linkers typically undergo intramolecular cleavage with a half-life between about 10 minutes and about 24 hours in water at a pH of approximately 7.4. Preferably, the cleavage half-life is between about 20 minutes and about 4 hours in water at a pH of approximately 7.4. More preferably, the cleavage half-life is between about 30 minutes and about 2 hours in water at a pH of approximately 7.4.

Photo-illumination of the conjugate causes release of the 6-mercaptopurine by virtue of the nitro group that is ortho to the mercaptomethyl moiety. This approach finds utility in phototherapy methods as are known in the art, particularly for localizing drug activation to a selected area of the body.

Preferably, the cleavable linker contains first and second cleavable groups that can cooperate to cleave the polymer from the biologically active agent, as illustrated by the following approaches. That is, the cleavable linker contains a first cleavable group that is distal to the agent, and a second cleavable group that is proximal to the agent, such that cleavage of the first cleavable group yields a linker-agent conjugate containing a nucleophilic moiety capable of reacting intramolecularly to cleave the second cleavable group, thereby releasing the agent from the linker and polymer.

Figure 5C shows a conjugate (III) containing a transport polymer T linked to the anticancer agent, 5-fluorouracil (5FU). Here, the linkage is provided by a modified lysyl residue. The transport polymer is linked to the α-amino group, and the 5-fluorouracil is linked via the α-carbonyl. The lysyl ε-amino group has been modified to a carbamate ester of *o*-hydroxymethyl nitrobenzene, which comprises a first, photolabile cleavable group in the conjugate. Photo-illumination severs the nitrobenzene moiety from the conjugate, leaving a carbamate that also rapidly decomposes to give the free α-amino group, an effective nucleophile. Intramolecular reaction of the ε-amino group with the amide linkage to the 5-fluorouracil group leads to cyclization with release of the 5-fluorouracil group.

Figure 5D illustrates a conjugate (IV) containing a delivery-enhancing transporter T linked to 2'-oxygen of the anticancer agent, paclitaxel. The linkage is provided by a linking moiety that includes (i) a nitrogen atom attached to the delivery-enhancing transporter, (ii) a phosphate monoester located para to the nitrogen atom, and (iii) a carboxymethyl group meta to the nitrogen atom, which is joined to the 2'-oxygen of paclitaxel by a carboxylate ester linkage. Enzymatic cleavage of the phosphate group from the conjugate affords a free phenol hydroxyl group. This nucleophilic group then reacts intramolecularly with the carboxylate ester to release free paclitaxel, fully capable of binding to its biological target. Example 9C of PCT application US98/10571 describes a synthetic protocol for preparing this type of conjugate.

Figure 5E illustrates yet another approach wherein a delivery-enhancing transporter is linked to a biologically active agent, *e.g.*, paclitaxel, by an aminoalkyl

administration. Thus, administration can be, for example, intravenous, topical, subcutaneous, transcutaneous, intramuscular, oral, intra-joint, parenteral, peritoneal, intranasal, or by inhalation. Suitable sites of administration thus include, but are not limited to, skin, bronchial, gastrointestinal, anal, vaginal, eye, and ear. The formulations may take the form of solid, semi-solid, lyophilized powder, or liquid dosage forms, such as, for example, tablets, pills, capsules, powders, solutions, suspensions, emulsions, suppositories, retention enemas, creams, ointments, lotions, aerosols or the like, preferably in unit dosage forms suitable for simple administration of precise dosages.

The compositions typically include a conventional pharmaceutical carrier or excipient and may additionally include other medicinal agents, carriers, adjuvants, and the like. Preferably, the composition will be about 5% to 75% by weight of a compound or compounds of the invention, with the remainder consisting of suitable pharmaceutical excipients. Appropriate excipients can be tailored to the particular composition and route of administration by methods well known in the art, *e.g.*, *REMINGTON'S PHARMACEUTICAL SCIENCES*, 18TH ED., Mack Publishing Co., Easton, PA (1990).

For oral administration, such excipients include pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, gelatin, sucrose, magnesium carbonate, and the like. The composition may take the form of a solution, suspension, tablet, pill, capsule, powder, sustained-release formulation, and the like.

In some embodiments, the pharmaceutical compositions take the form of a pill, tablet or capsule, and thus, the composition can contain, along with the biologically active conjugate, any of the following: a diluent such as lactose, sucrose, dicalcium phosphate, and the like; a disintegrant such as starch or derivatives thereof; a lubricant such as magnesium stearate and the like; and a binder such a starch, gum acacia, polyvinylpyrrolidone, gelatin, cellulose and derivatives thereof.

The active compounds of the formulas may be formulated into a suppository comprising, for example, about 0.5% to about 50% of a compound of the invention, disposed in a polyethylene glycol (PEG) carrier (e.g., PEG 1000 [96%] and PEG 4000 [4%]).

Liquid compositions can be prepared by dissolving or dispersing compound (about 0.5% to about 20%), and optional pharmaceutical adjuvants in a carrier, such as, for

comprising mixtures of D and L-residues have intermediate stabilities. Homo-D-polymers are generally preferred.

A. Application to Skin

The delivery-enhancing transporters of the invention make possible the delivery of biologically active and diagnostic agents across the skin. Surprisingly, the transporters can deliver an agent across the stratum corneum, which previously had been a nearly impenetrable barrier to drug delivery. The stratum corneum, the outermost layer of the skin, is composed of several layers of dead, keratin-filled skin cells that are tightly bound together by a "glue" composed of cholesterol and fatty acids. Once the agents are delivered through the stratum corneum by the transporters of the invention, the agents can enter the viable epidermis, which is composed of the stratum granulosum, stratum lucidum and stratum germinativum which, along with the stratum corneum, make up the epidermis. Delivery in some embodiments of the invention is through the epidermis and into the dermis, including one or both of the papillary dermis and the reticular dermis.

This ability to obtain penetration of one or more layers of the skin can greatly enhance the efficacy of compounds such as antibacterials, antifungals, antivirals, antiproliferatives, immunosuppressives, vitamins, analgesics, hormones, and the like. Numerous such compounds are known to those of skill in the art (*see, e.g.*, Hardman and Limbird, *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, 1996).

In some embodiments, the agent is delivered into a blood vessel that is present in the epithelial tissue, thus providing a means for delivery of the agent systemically. Delivery can be either intrafollicular or interfollicular, or both. Pretreatment of the skin is not required for delivery of the conjugates.

In other embodiments, the delivery-enhancing transporters are useful for delivering cosmetics and agents that can treat skin conditions. Target cells in the skin that are of interest include, for example, fibroblasts, epithelial cells and immune cells. For example, the transporters provide the ability to deliver compounds such as antiinflammatory agents to immune cells found in the dermis.

Glucocorticoids (adrenocorticoid steroids) are among the compounds for which delivery across skin can be enhanced by the delivery-enhancing transporters of the

Mollinson *et al.*, *Current Pharm. Design* 4(5):367-380 (1998); U.S. Patent Nos. 5,612,350; 5,599,927; 5,604,294;5,990,131; 5,561,140; 5,859,031; 5,925,649; 5,994,299; 6,004,973 and 5,508,397). Cyclosporins include cyclosporin A, B, C, D , G and M. *See, e.g.*, U.S. Patent No. 6,007,840; and 6,004,973. .For example, such compounds are useful in treating psoriasis, eczema (including atopic dermatitis, contact dermatitis, allergic dermatitis) and alopecia areata.

The delivery-enhancing transporters can be conjugated to agents that are useful for treating conditions such as lupus erythematosus (both discoid and systemic), cutaneous dermatomyositis, porphyria cutanea tarda and polymorphous light eruption. Agents useful for treating such conditions include, for example, quinine, chloroquine, hydroxychloroquine, and quinacrine.

The delivery-enhancing transporters of the invention are also useful for transdermal delivery of antiinfective agents. For example, antibacterial, antifungal and antiviral agents can be conjugated to the delivery-enhancing transporters. Antibacterial agents are useful for treating conditions such as acne, cutaneous infections, and the like. Antifungal agents can be used to treat tinea corporis, tinea pedis, onychomycosis, candidiasis, tinea versicolor, and the like. Because of the delivery-enhancing properties of the conjugates, these conjugates are useful for treating both localized and widespread infections. Antifungal agents are also useful for treating onychomycosis. Examples of antifungal agents include, but are not limited to, azole antifungals such as itraconazole, myconazole and fluconazole. Examples of antiviral agents include, but are not limited to, acyclovir, famciclovir, and valacyclovir. Such agents are useful for treating viral diseases, e.g., herpes.

Another example of a biologically active agent for which enhancement of delivery by conjugation to the delivery-enhancing transporters of the invention is desirable are the antihistamines. These agents are useful for treating conditions such as pruritus due to urticaria, atopic dermatitis, contact dermatitis, psoriasis, and many others. Examples of such reagents include, for example, terfenadine, astemizole, lorotadine, cetirizine, acrivastine, temelastine, cimetidine, ranitidine, famotidine, nizatidine, and the like. Tricyclic antidepressants can also be delivered using the delivery-enhancing transporters of the invention.

on invasive bacteria, such as *Shigella, Salmonella*, and *Yersinia*. Such compounds include, for example, norfloxacin, ciprofloxacin, trimethoprim, sulfamethyloxazole, and the like.

Anti-neoplastic agents can also be conjugated to the delivery-enhancing transporters of the invention and administered by the gastrointestinal route. These include, for example, cisplatin, methotrexate, taxol, fluorouracil, mercaptopurine, donorubicin, bleomycin, and the like.

C. Respiratory Tract Administration

The delivery-enhancing transporters of the invention can also used to enhance administration of drugs through the respiratory tract. The respiratory tract, which includes the nasal mucosa, hypopharynx, and large and small airway structures, provides a large mucosal surface for drug absorption. The enhanced penetration of the conjugated agents into and across one or more layers of the epithelial tissue that is provided by the delivery-enhancing transporters of the invention results in amplification of the advantages that respiratory tract delivery has over other delivery methods. For example, lower doses of an agent are often needed to obtain a desired effect, a local therapeutic effect can occur more rapidly, and systemic therapeutic blood levels of the agent are obtained quickly. Rapid onset of pharmacological activity can result from respiratory tract administration. Moreover, respiratory tract administration generally has relatively few side effects.

The transporters of the invention can be used to deliver biological agents that are useful for treatment of pulmonary conditions. Examples of conditions treatable by nasal administration include, for example, asthma. These compounds include antiinflammatory agents, such as corticosteroids, cromolyn, and nedocromil, bronchodialators such as β2-selective adronergic drugs and theophylline, and immunosuppressive drugs (*e.g.*, cyclosporin and FK506). Other conditions include, for example, allergic rhinitis (which can be treated with glucocorticoids), and chronic obstructive pulmonary disease (emphysema). Other drugs that act on the pulmonary tissues and can be delivered using the transporters of the invention include beta-agonists, mast cell stabilizers, antibiotics, antifungal and antiviral agents, surfactants, vasoactive drugs, sedatives and hormones.

Respiratory tract administration is useful not only for treatment of pulmonary conditions, but also for delivery of drugs to distant target organs via the circulatory system.

agents are also contemplated (*e.g.*, Langston (1997) *DDT* 2:255; Giannis *et al.* (1997) *Advances Drug Res.* 29:1). Also, the invention is advantageous for delivering small organic molecules that have poor solubilities in aqueous liquids, such as serum and aqueous saline. Thus, compounds whose therapeutic efficacies are limited by their low solubilities can be administered in greater dosages according to the present invention, and can be more efficacious on a molar basis in conjugate form, relative to the non-conjugate form, due to higher uptake levels by cells.

Since a significant portion of the topological surface of a small molecule is often involved, and therefore required, for biological activity, the small molecule portion of the conjugate in particular cases may need to be severed from the attached delivery-enhancing transporter and linker moiety (if any) for the small molecule agent to exert biological activity after crossing the target epithelial tissue. For such situations, the conjugate preferably includes a cleavable linker for releasing free drug after passing through an epithelial tissue.

Figure 5D and Figure 5E are illustrative of another aspect of the invention, comprising taxane- and taxoid anticancer conjugates which have enhanced trans-epithelial tissue transport rates relative to corresponding non-conjugated forms. The conjugates are particularly useful for inhibiting growth of cancer cells. Taxanes and taxoids are believed to manifest their anticancer effects by promoting polymerization of microtubules (and inhibiting depolymerization) to an extent that is deleterious to cell function, inhibiting cell replication and ultimately leading to cell death.

The term "taxane" refers to paclitaxel (Figure 5F, R' = acetyl, R" = benzyl) also known under the trademark "TAXOL") and naturally occurring, synthetic, or bioengineered analogs having a backbone core that contains the A, B, C and D rings of paclitaxel, as illustrated in Figure 5G. Figure 5F also indicates the structure of "TAXOTERE™" (R' = H, R" = BOC), which is a somewhat more soluble synthetic analog of paclitaxel sold by Rhone-Poulenc. "Taxoid" refers to naturally occurring, synthetic or bioengineered analogs of paclitaxel that contain the basic A, B and C rings of paclitaxel, as shown in Figure 5H. Substantial synthetic and biological information is available on syntheses and activities of a variety of taxane and taxoid compounds, as reviewed in Suffness (1995) *Taxol: Science and Applications*, CRC Press, New York, NY, pp. 237-239,

C. Macromolecules

The enhanced transport methods of the invention are particularly suited for enhancing transport into and across one or more layers of an epithelial or endothelial tissue for a number of macromolecules, including, but not limited to proteins, nucleic acids, polysaccharides, and analogs thereof. Exemplary nucleic acids include oligonucleotides and polynucleotides formed of DNA and RNA, and analogs thereof, which have selected sequences designed for hybridization to complementary targets (*e.g.*, antisense sequences for single- or double-stranded targets), or for expressing nucleic acid transcripts or proteins encoded by the sequences. Analogs include charged and preferably uncharged backbone analogs, such as phosphonates (preferably methyl phosphonates), phosphoramidates (N3' or N5'), thiophosphates, uncharged morpholino-based polymers, and protein nucleic acids (PNAs). Such molecules can be used in a variety of therapeutic regimens, including enzyme replacement therapy, gene therapy, and anti-sense therapy, for example.

By way of example, protein nucleic acids (PNA) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone. The backbone consists of N-(2-aminoethyl)glycine units to which the nucleobases are attached. PNAs containing all four natural nucleobases hybridize to complementary oligonucleotides obeying Watson-Crick base-pairing rules, and is a true DNA mimic in terms of base pair recognition (Egholm *et al.* (1993) *Nature* 365:566-568. The backbone of a PNA is formed by peptide bonds rather than phosphate esters, making it well-suited for anti-sense applications. Since the backbone is uncharged, PNA/DNA or PNA/RNA duplexes that form exhibit greater than normal thermal stability. PNAs have the additional advantage that they are not recognized by nucleases or proteases. In addition, PNAs can be synthesized on an automated peptides synthesizer using standard t-Boc chemistry. The PNA is then readily linked to a transport polymer of the invention.

Examples of anti-sense oligonucleotides whose transport into and across epithelial and endothelial tissues can be enhanced using the methods of the invention are described, for example, in U.S. Patent 5,594,122. Such oligonucleotides are targeted to treat human immunodeficiency virus (HIV). Conjugation of a transport polymer to an anti-sense oligonucleotide can be effected, for example, by forming an amide linkage between the peptide and the 5'-terminus of the oligonucleotide through a succinate linker, according to

In another embodiment, the invention is useful for delivering immunospecific antibodies or antibody fragments to the cytosol to interfere with deleterious biological processes such as microbial infection. Recent experiments have shown that intracellular antibodies can be effective antiviral agents in plant and mammalian cells (*e.g.*, Tavladoraki *et al.* (1993) *Nature* 366:469; and Shaheen *et al.* (1996) *J. Virol.* 70:3392. These methods have typically used single-chain variable region fragments (scFv), in which the antibody heavy and light chains are synthesized as a single polypeptide. The variable heavy and light chains are usually separated by a flexible linker peptide (*e.g.*, of 15 amino acids) to yield a 28 kDa molecule that retains the high affinity ligand binding site. The principal obstacle to wide application of this technology has been efficiency of uptake into infected cells. But by attaching transport polymers to scFv fragments, the degree of cellular uptake can be increased, allowing the immunospecific fragments to bind and disable important microbial components, such as HIV Rev, HIV reverse transcriptase, and integrase proteins.

D. Peptides

Peptides to be delivered by the enhanced transport methods described herein include, but should not be limited to, effector polypeptides, receptor fragments, and the like. Examples include peptides having phosphorylation sites used by proteins mediating intracellular signals. Examples of such proteins include, but are not limited to, protein kinase C, RAF-1, p21Ras, NF-κB, C-JUN, and cytoplasmic tails of membrane receptors such as IL-4 receptor, CD28, CTLA-4, V7, and MHC Class I and Class II antigens.

When the delivery-enhancing transporter is also a peptide, synthesis can be achieved either using an automated peptide synthesizer or by recombinant methods in which a polynucleotide encoding a fusion peptide is produced, as mentioned above.

EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

labeled streptavidin at 30μg/ml for thirty minutes, washed with PBS, counterstained with propidium iodide (1μg/ml) for two minutes, and the section was mounted with Vectashield™ mounting media. Slides were analyzed by fluorescent microscopy. Parallel studies were done using streptavidin-horse radish peroxidase rather than fluorescein-streptavidin. The biotinylated peptide was visualized by treatment of the sections with the horseradish peroxidase substrate diaminobenzadine, and visualization with light microscopy.

Results

Biotinylated arginine heptamer crossed into and across the epidermis and into the dermis. The cytosol and nuclei of all cells in the field were fluorescent, indicating penetration into virtually every cell of the nude mouse skin in the section. The staining pattern was consistent with unanticipated transport that was both follicular and interfollicular. In addition, positive cells were apparent in papillary and reticular dermis. In contrast, no staining was apparent in mice treated with biotin alone, or phosphate buffered saline alone.

Example 2

Penetration of biotinylated polymers of D-arginine into the skin of normal Balb/C mice

Varying concentrations (1mM-100μM) of a heptamer of D-arginine with biotin covalently attached to the amino terminus using an amino caproic acid spacer (bio r7), dissolved in PBS, were applied to a skin of the groin of an anesthetized Balb/C mice. Sample (100μl) was applied as a liquid within excipient and prevented from dispersing by a Vaseline™ barrier and allowed to penetrate for thirty minutes. At the end of this period animal was sacrificed, the relevant section of skin was excised, embedded in mounting medium (OCT) and frozen. Frozen sections were cut using a cryostat, collected on slides, and stained with fluorescently labeled streptavidin (Vector Laboratories, Burlingame, CA) as described in Example 1. Slides were analyzed by fluorescent microscopy.

Results

As with the skin from nude mice, biotinylated arginine heptamer crossed into and across the epidermis and into the dermis. The cytosol and nuclei of all cells in the field were fluorescent, indicating penetration into virtually every cell of the nude mouse skin in

Example 4

Increased penetration of biotinylated polymers of D-arginine into skin of nude mouse using plastic wrap or a lotion excipient.

Varying concentrations (1mM-100μM) of a heptamer of D-arginine with biotin covalently attached to the amino terminus using an amino caproic acid spacer (bio r7), dissolved in PBS, and mixed with an equal volume of Lubriderm™. The lotion mixture was then applied to the back of nude mice and allowed to penetrate for thirty, sixty, and 120 minutes. Alternatively, sample (100μl) was applied as a liquid without excipient and prevented from evaporating by wrapping plastic wrap over the sample sealed with Vaseline™. The samples were allowed to penetrate for thirty, sixty, and 120 minutes. At the end of this period animal was sacrificed, the relevant section of skin was excised, embedded in mounting medium (OCT) and frozen. Frozen sections were cut using a cryostat, collected on slides, and stained with fluorescently labeled streptavidin (Vector Laboratories, Burlingame, CA) as described in Example 1. Slides were analyzed by fluorescent microscopy.

Results

Both lotion and plastic wrap resulted in increased uptake compared with staining without excipient. Lotion was more effective than plastic wrap in enhancing uptake of the conjugate. Biotinylated arginine pentamers crossed into and across several skin layers, reaching both the cytosol and nuclei of epidermal cell layers, both follicular and interfollicular. In addition, positive cells were apparent in papillary and reticular dermis.

Example 5

Penetration of cyclosporin conjugated to a biotinylated pentamer, heptamer, and nonamer of D-arginine into the skin of nude mice.

Methods

A. Linking cyclosporin to delivery-enhancing transporters

1. Preparation of the α-chloroacetyl Cyclosporin A derivative.

The α-chloroacetyl cyclosporin A derivative was prepared as shown in Figure 1. Cyclosporin A (152.7 mg, 127 μmol) and chloroacetic acid anhydride (221.7 mg; 1300

B. Analysis of transport across skin

Varying concentrations (1mM-100μM) of cyclosporin conjugated to either biotinylated pentamer, heptamer, or nonamers of D-arginine (bio r5, r7, or r9), dissolved in PBS, were applied to the back of nude mice. Samples (100μl) were applied as a liquid within excipient and prevented from dispersing by a Vaseline™ barrier and allowed to penetrate for thirty, sixty, and 120 minutes. At the end of this period animal was sacrificed, the relevant section of skin was excised, embedded in mounting medium (OCT) and frozen. Frozen sections were cut using a cryostat, collected on slides, and stained with fluorescently labeled streptavidin (Vector Laboratories, Burlingame, CA) as described in Example 1. Slides were analyzed by fluorescent microscopy

Results

The conjugates of cyclosporin with biotinylated heptamers and nonamers of D-arginine effectively entered into and across the epidermis and into the dermis of the skin of nude mice. In contrast, very little uptake was seen using a conjugate between a pentamer of arginine and cyclosporin, and no staining was seen with a PBS control. The cytosol and nuclei of all cells in the field were fluorescent, indicating penetration into and through the epidermis and dermis. The staining pattern was consistent with unanticipated transport that was both follicular and interfollicular. In addition, positive cells were apparent in papillary and reticular dermis. These results demonstrate remarkable uptake only when sufficient guanidinyl groups are included in the delivery-enhancing transporter.

Example 6

Demonstration that a D-arginine heptamer can penetrate human skin.

Human and murine skin differ significantly in a number of ways, with human epidermis being considerably thicker. To determine if the D-arginine heptamers/cyclosporin A (r7 CsA) conjugate could also penetrate human skin, biotin r7 CsA was applied to full thickness human skin grafted onto the back of a SCID mouse. As in murine skin, conjugated cyclosporin A penetrated human epidermis and dermis. Fluorescence was observed in both the cytosol and the nuclei of cells in tissue exposed to biotinylated peptides alone, but in sections stained with biotin r7 CsA the majority of fluorescence was cytosolic, consistent with r7 CsA binding to cyclosporin A's known cytoplasmic targets.

Example 8

Synthesis, *in vitro* and *in vivo* activity of a releasable conjugate of a short oligomer of arginine and CsA

Modification of the 2° alcohol of Cyclosporin A results in significant loss of its biological activity. *See, e.g.*, R. E. Handschumacher, *et al.*, *Science* 226, 544-7 (1984). Consequently, to ensure release of free Cyclosporin A from its conjugate after transport into cells, Cyclosporin A was conjugated to an oligo-arginine transporter through a pH sensitive linker as shown in Figure 10. The resultant conjugate is stable at acidic pH but at pH>7 it undergoes an intramolecular cyclization involving addition of the free amine to the carbonyl adjacent to Cyclosporin A (Figure 6), which results in the release of unmodified Cyclosporin A.

Another modification in the design of the releasable conjugate was the use of L-arginine (R), and not D-arginine (r) in the transporter. While the oligo-D-arginine transporters were used for the histological experiments to ensure maximal stability of the conjugate and therefore accuracy in determining its location through fluorescence, oligomers of L- arginine were incorporated into the design of the releasable conjugate to minimize its biological half-life. Consistent with its design, the resultant releasable conjugate was shown to be stable at acidic pH, but labile at physiological pH in the absence of serum. This releasable Cyclosporin A conjugate's half-life in pH 7.4 PBS was 90 minutes.

Results

The releasable guanidino-heptamer conjugate of Cyclosporin A was shown to be biologically active by inhibiting IL-2 secretion by the human T cell line, Jurkat, stimulated with PMA and ionomycin *in vitro*. *See* R. Wiskocil, *et al.*, *J Immunol* 134, 1599-603 (1985). The conjugate was added 12 hours prior to the addition of PMA/ionomycin and dose dependent inhibition was observed by the releasable R7 CsA conjugate. This inhibition was not observed with a nonreleasable analog (Figure 6) that differed from the releasable conjugate by retention of the t-Boc protecting group, which prevented cyclization and resultant release of the active drug. The EC_{50} of the releasable R7 cyclosporin conjugate was approximately two fold higher than CsA dissolved in alcohol and added at the same time as the releasable conjugate.

water/acetonitrile gradient. Treatment with TFA resulted in loss of Cu^{2+} ion which needed to be reinserted.

DTPA-aca-R7-CO2H (10 mg, 0.0063 mmol) and copper sulfate (1.6 mg, 0.0063 mmol) were dissolved in water (1 mL). Let gently stir for 18 h and lyophilized to provide product as a white powder (10 mg).

2. Analysis of transport across skin

Metal diethylenetriaminepentaacetic acid (DTPA) complexes were formed by mixing equimolar amounts of metal salts with DTPA in water for 18 hours. At the end of this time, the solutions were centrifuged, frozen and lyophilized. The dried powder was characterized by mass spectrometry and used in solid phase peptide synthesis. The metal-DTPA complexes were attached to polymers of D- or L-arginine that were still attached to solid-phase resin used in peptide synthesis. The metal-DTPA complexes were attached using an aminocaproic acid spacer. The solid phase peptide synthesis techniques were described in Example 1, with the exception that cleavage of the peptide- DTPA -metal complex in trifluoroacetic acid released the metal. The metal is replaced after HPLC purification and lyophilization of the peptide- DTPA complex. Replacement of the metal involved incubation of equimolar amounts of the metal salt with the peptide-aminocaproic acid- DTPA complex and subsequent lyophilization.

Varying concentrations (1 μM to 1 mM) of the Cu- DTPA -aca-r7 complex were applied to the abdominal region of nude mice for 15, 30 and 45 minutes. As controls, an equimolar amount of the Cu- DTPA complex was spotted onto the abdominal region. At the end of the incubation period, the samples were simply wiped off and intense blue color was apparent on the skin where the Cu- DTPA -aca-r7 complex was spotted and not where the Cu- DTPA alone was spotted. In the case of the application of 1 mM, visible blue dye was seen for three days, decreasing with time, but being apparent for the full period.

Varying concentrations (1 μM to 1 mM) of the Gd- DTPA -aca-r7 complex are injected into the tail vein of BALB/c mice in 100 μl. Distribution of the Gd is observed in real time using magnetic resonance imaging. Distribution of the dye is apparent throughout the bloodstream, entering liver, spleen, kidney, and heart. When injected into the carotid artery of rabbits, the dye is seen to cross the blood brain barrier.

streptavidin (Vector Laboratories, Burlingame, CA) as described in Example 1. Slides were analyzed by fluorescent microscopy.

Results

The conjugates of hydrocortisone with biotinylated heptamers of D-arginine effectively entered into and across the epidermis and into the dermis of the skin of nude mice. In contrast, very little uptake was seen using a conjugate between a pentamer of arginine and hydrocortisone, and no staining was seen with a PBS control. The cytosol and nuclei of all cells in the field were fluorescent, indicating penetration into and through the epidermis and dermis. The staining pattern was consistent with unanticipated transport that was both follicular and interfollicular. In addition, positive cells were apparent in papillary and reticular dermis. These results demonstrate remarkable uptake only when sufficient guanidinyl groups are included in the delivery-enhancing transporter.

Example 11

Penetration of taxol conjugated to a biotinylated pentamer, heptamer, and nonamer of D-arginine into the skin of nude mice

Methods

1. Conjugation of C-2' activated taxol derivatives to biotin-labeled peptides

Synthesis of C-2' Derivatives

Taxol (48.7 mg, 57.1 μmol) was dissolved in CH_2Cl_2 (3.0 mL) under an N_2-atmosphere. The solution was cooled to 0°C. A stock solution of the chloroformate of benzyl-(*p*-hydroxy benzoate) (200 mmol, in 2.0 mL CH_2Cl_2 - freshly prepared from benzyl-(*p*-hydroxy benzoate) and diphosgene) was added at 0°C and stirring at that temperature was continued for 5 hours, after which the solution was warmed to room temperature (Figure 12). Stirring was continued for additional 10 hours. The solvents were removed *in vacuo* and the crude material was purified by flash chromatography on silica gel (eluent: EtOAc/hexanes 30%-70%) yielding the desired taxol C-2' carbonate (36.3 mg, 32.8 μmol, 57.4%).

The solvent was removed *in vacuo* and the resultant crude material was purified by flash chromatography on silica gel (eluent: EtOAc/ hexanes 40%-80%) yielding the desired product (13.6 mg, 12.2 μmol, 69%).

The activated taxol derivative (14.0 mg, 12.6 μmol) and the peptide (30.6 mg, 15.1 μmol) were dissolved in DMF (3.0 mL) under an N_2-atmosphere (Figure 15C). Diisopropylethylamine (1.94 mg, 15.1 μmol) was added as a stock solution in DMF (0.1 mL), followed by DMAP (0.76 mg, 6.3 μmol) as a stock solution in DMF 0.1 mL). Stirring at room temperature was continued until all the starting material was consumed. After 20 hours the solvent was removed *in vacuo*. The crude material was dissolved in water and purified by RP-HPLC (eluent: water/MeCN * TFA) yielding the two depicted taxol conjugates in a ration of 1:6 (carbonate vs carbamate, respectively).

2. ***Analysis of transport across skin***

Varying concentrations (1mM-100μM) of taxol conjugated to either biotinylated pentamer, heptamer, or nonamers of D-arginine (bio r5, r7, or r9), dissolved in PBS, were applied to the back of nude mice. Samples (100μl) were applied as a liquid within excipient and prevented from dispersing by a Vaseline™ barrier and allowed to penetrate for thirty, sixty, and 120 minutes. At the end of this period animal was sacrificed, the relevant section of skin was excised, embedded in mounting medium (OCT) and frozen. Frozen sections were cut using a cryostat, collected on slides, and stained with fluorescently labeled streptavidin (Vector Laboratories, Burlingame, CA) as described in Example 1. Slides were analyzed by fluorescent microscopy.

Results

The conjugates of taxol with biotinylated heptamers and nonamers of D-arginine effectively entered into and across the epidermis and into the dermis of the skin of nude mice. In contrast, very little uptake was seen using a conjugate between a pentamer of arginine and taxol, and no staining was seen with a PBS control. The cytosol and nuclei of all cells in the field were fluorescent, indicating penetration into and through the epidermis and dermis. The staining pattern was consistent with unanticipated transport that was both follicular and interfollicular. In addition, positive cells were apparent in papillary and

Linkage of Taxol to Delivery-enhancing transporter

The peptide (47.6 mg, 22.4 μmol) was dissolved in DMF (1.0 mL) under an N_2-atmosphere. DIEA (2.8 mg, 22.4 μmol) was added. A solution of taxol-2'-chloroacetate (20.8 mg, 22.4 μmol) in DMF (1.0 mL) was added. Stirring at room temperature was continued for 6 hours. Water containing 0.1% TFA (1.0 mL) was added, the sample was frozen and the solvents were lyophilized. The crude material was purified by RP-HPLC (eluent: water/MeCN *0.1%TFA: 85% - 15%). A schematic of this reaction is shown in Figure 18.

Synthesis of Related Conjugates

Using the conjugation conditions outlined above, the three additional conjugates shown in were synthesized.

Cytotoxicity Assay

The taxol conjugates were tested for cytotoxicity in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) dye reduction. Results, which are shown in Figure 20, demonstrate that the taxol conjugated to r7 with a readily pH-releasable linker (CG 1062; R=Ac in the structure shown in Figure 19) is significantly more cytotoxic than either taxol alone or taxol conjugated to r7 with a less-readily pH-releasable linker (CG 1040; R=H in the structure shown in Figure 19).

Example 13

Structure-Function Relationships of Fluorescently-Labeled Peptides Derived from Tat_{49-57}

Methods

General. Rink amide resin and Boc_2O were purchased from Novabiochem. Diisopropylcarbodiimide, bromoacetic acid, fluorescein isothiocyanate (FITC-NCS), ethylenediamine, 1,3-diaminopropane, 1,4-diaminobutane, 1,6-diaminohexane, *trans*-1,6-diaminocyclohexane, and pyrazole-1-carboxamidine were all purchased from Aldrich®. All solvents and other reagents were purchased from commercial sources and used without further purification. The mono-Boc amines were synthesized from the commercially

each of these were used for cellular uptake experiments. Varying amounts of arginine and oligomers of guanidine-substituted peptoids were added to approximately 3 x 10^6 cells in 2% FCS/PBS (combined total of 200 μL) and placed into microtiter plates (96 well) and incubated for varying amounts of time at 23 °C or 4 °C. The microtiter plates were centrifuged and the cells were isolated, washed with cold PBS (3 x 250 μL), incubated with 0.05% trypsin/0.53 mM EDTA at 37 °C for 5 min, washed with cold PBS, and resuspended in PBS containing 0.1% propidium iodide. The cells were analyzed using fluorescent flow cytometry (FACScan, Becton Dickinson) and cells staining with propidium iodide were excluded from the analysis. The data presented is the mean fluorescent signal for the 5000 cells collected.

Inhibition of Cellular Uptake with Sodium Azide. The assays were performed as previously described with the exception that the cells used were preincubated for 30 min with 0.5% sodium azide in 2% FCS/PBS buffer prior to the addition of fluorescent peptides and the cells were washed with 0.5% sodium azide in PBS buffer. All of the cellular uptake assays were run in parallel in the presence and absence of sodium azide.

Cellular Uptake Kinetics Assay. The assays were performed as previously described except the cells were incubated for 0.5, 1, 2, and 4 min at 4 °C in triplicate in 2% FCS/PBS (50 μl) in microtiter plates (96 well). The reactions were quenched by diluting the samples into 2% FCS/PBS (5 mL). The assays were then worked up and analyzed by fluorescent flow cytometry as previously described.

Results

To determine the structural requirements for the cellular uptake of short arginine-rich peptides, a series of fluorescently-labeled truncated analogues of Tat_{49-57} were synthesized using standard solid-phase chemistry. *See, e.g.*, Atherton, E.*et al.* SOLID-PHASE PEPTIDE SYNTHESIS (IRL: Oxford, Engl. 1989). A fluorescein moiety was attached via an aminohexanoic acid spacer on the amino termini. The ability of these fluorescently labeled peptides to enter Jurkat cells was then analyzed using fluorescent activated cell sorting (FACS) (). The peptide constructs tested were Tat_{49-57} (Fl-ahx-RKKRRQRRR): Tat_{49-56} (Fl-ahx-RKKRRQRR), Tat_{49-55} (Fl-ahx-RKKRRQR), Tat_{50-57} (Fl-ahx-KKRRQRRR), and

arginine and two lysines found in Tat_{49-57} demonstrated enhanced cellular uptake. Thus, residues at the amine terminus appear to be important and that arginines are more effective than lysines for internalization. The improved cellular uptake of the unnatural *d*-peptides is most likely due to their increased stability to proteolysis in 2% FCS (fetal calf serum) used in the assays. When serum was excluded, the *d*- and *l*-peptides were equivalent as expected.

These initial results indicated that arginine content is primarily responsible for the cellular uptake of Tat_{49-57}. Furthermore, these results were consistent with our previous results where we demonstrated that short oligomers of arginine were more effective at entering cells then the corresponding short oligomers of lysine, ornithine, and histidine. What had not been established was whether arginine homo-oligomers are more effective than Tat_{49-57}. To address this point, Tat_{49-57} was compared to the *l*-arginine (R5-R9) and *d*-arginine (r5-r9) oligomers. Although Tat_{49-57} contains eight cationic residues, its cellular internalization was between that of R6 and R7 (Figure 24) demonstrating that the presence of six arginine residues is the most important factor for cellular uptake. Significantly, conjugates containing 7-9 arginine residues exhibited better uptake than Tat_{49-57}.

To quantitatively compare the ability of these arginine oligomers and Tat_{49-57} to enter cells, Michaelis-Menton kinetic analyses were performed. The rates of cellular uptake were determined after incubation (3 °C) of the peptides in Jurkat cells for 30, 60, 120, and 240 seconds (Table 1). The resultant K_m values revealed that r9 and R9 entered cells at rates approximately 100-fold and 20-fold faster than Tat_{47-59} respectively. For comparison, $Antennapedia_{43-58}$ was also analyzed and was shown to enter cells approximately 2-fold faster than Tat_{47-59}, but significantly slower than r9 or R9.

Table 1: Michaelis-Menton kinetics: $Antennapedia_{43-58}$ (Fl-ahx-RQIKIWFQNRRMKWKK).

peptide	$K_m(\mu M)$	V_{max}
Tat_{49-57}	770	0.38
$Antennapedia_{43-58}$	427	0.41
R9	44	0.37
r9	7.6	0.38

equivalents per amine residue) and heated to 50° C for 24-48 hr. The crude mixture was then acidified with TFA (0.5 mL) and directly purified by reverse-phase HPLC (H_2O/CH_3CN in 0.1% TFA). The products were characterized by electrospray mass spectrometry and isolated by lyophilization and further purified by reverse-phase HPLC. The purity of the guanidine-substituted peptoids was >95% as determined by analytical reverse-phase HPLC (H_2O/CH_3CN in 0.1% TFA).

Results

Utilizing the structure-function relationships that had been determined for the cellular uptake of Tat_{47-59}, we designed a set of polyguanidine peptoid derivatives that preserve the 1,4 backbone spacing of side chains of arginine oligomers, but have an oligo-glycine backbone devoid of stereogenic centers. These peptoids incorporating arginine-like side chains on the amide nitrogen were selected because of their expected resistance to proteolysis, and potential ease and significantly lower cost of synthesis (Simon *et al.*, *Proc. Natl. Acad. Sci. USA* 89:9367-9371 (1992); Zuckermann, *et al.*, *J. Am. Chem. Soc.* 114:10646-10647 (1992). Furthermore, racemization, frequently encountered in peptide synthesis, is not a problem in peptoid synthesis; and the "sub-monomer" peptoid approach allows for facile modification of side-chain spacers. Although the preparation of an oligurea and peptoid-peptide hybrid (Hamy, *et al*, *Proc. Natl. Acad. Sci. USA* 94:3548-3553 (1997)) derivatives of Tat_{49-57} have been previously reported, their cellular uptake was not explicitly studied.

The desired peptoids were prepared using the "sub-monomer" approach (Simon *et al.*; Zuckermann *et al.*) to peptoids followed by attachment of a fluorescein moiety via an aminohexanoic acid spacer onto the amine termini. After cleavage from the solid-phase resin, the fluorescently labeled polyamine peptoids thus obtained were converted in good yields (60-70%) into polyguanidine peptoids by treatment with excess pyrazole-1-carboxamidine (Bernatowicz, *et al.*, *J. Org. Chem.* 57:2497-2502 (1992) and sodium carbonate (as shown in Figure 25). Previously reported syntheses of peptoids containing isolated N-Arg units have relied on the synthesis of N-Arg monomers (5-7 steps) prior to peptoid synthesis and the use of specialized and expensive guanidine protecting groups (Pmc, Pbf) (Kruijtzer, *et al.* , *Chem. Eur. J.* 4:1570-1580 (1998); Heizmann, *et al. Peptide*

uptake of the corresponding heptamers and pentamers also showed the same relative trend. The longer side chains embodied in the *N*-hxg peptoids improved the cellular uptake to such an extent that the amount of internalization was comparable to the corresponding *d*-arginine oligomer containing one more guanidine residue (Figure 28). For example, the *N*-hxg7 peptoid showed comparable cellular uptake to r8.

To address whether the increase in cellular uptake was due to the increased length of the side chains or due to their hydrophobic nature, a set of peptoids was synthesized containing cyclohexyl side chains. These are referred to as the *N*-chg5,7,9 peptoids. These contain the same number of side chain carbons as the N-hxg peptoids but possess different degrees of freedom. Interestingly, the *N*-chg peptoid showed much lower cellular uptake activity than all of the previously assayed peptoids, including the *N*-etg peptoids (Figure 29). Therefore, the conformational flexibility and sterically unencumbered nature of the straight chain alkyl spacing groups is important for efficient cellular uptake.

DISCUSSION

The nona-peptide, Tat_{49-57}, has been previously shown to efficiently translocate through plasma membranes. The goal of this research was to determine the structural basis for this effect and use this information to develop simpler and more effective molecular transporters. Toward this end, truncated and alanine substituted derivatives of Tat_{49-57} conjugated to a fluoroscein label was prepared. These derivatives exhibited greatly diminished cellular uptake compared to Tat_{49-57}, indicating that all of the cationic residues of Tat_{49-57} are required for efficient cellular uptake. When compared with our previous studies on short oligomers of cationic oligomers, these findings suggested that an oligomer of arginine might be superior to Tat_{49-57} and certainly more easily and cost effectively prepared. Comparison of short arginine oligomers with Tat_{49-57} showed that members of the former were indeed more efficiently taken into cells. This was further quantified for the first time bt Michaelis-Menton kinetics analysis which showed that the R9 and r9 oligomers had Km values 30-fold and 100-fold greater than that found for Tat_{49-57}.

Given the importance of the guanidino head group and the apparent insensitivity of the oligomer chirality revealed in our peptide studies, we designed and synthesized a novel series of polyguanidine peptoids. The peptoids *N*–arg5,7,9,

along that backbone are not required for cellular uptake, that the guanidino head group is superior to other cationic subunits, and most significantly, that an extension of the alkyl chain between the backbone and the head group provides superior transporters. In addition to better uptake performance, these novel peptoids offer several advantages over Tat_{49-57} including cost-effectiveness, ease of synthesis of analogs, and protease stability. These features along with their significant water solubility (>100 mg/mL) indicate that these novel peptoids could serve as effective transporters for the molecular delivery of drugs, drug candidates, and other agents into cells.

Example 16

Synthesis of Itraconazole-Transporter Conjugate

This Example provides one application of a general strategy for attaching a delivery-enhancing transporter to a compound that includes a triazole structure. The scheme, using attachment of itraconazole to an arginine (r7) delivery-enhancing transporter as an example, is shown in Figure 30. In the scheme, R is H or alkyl, n is 1 or 2, and X is a halogen.

The reaction involves making use of quaternization of a nitrogen in the triazole ring to attach an acyl group that has a halogen (*e.g.*, Br, Fl, I) or a methyl ester. Compound 3 was isolated by HPLC. Proton NMR in D_2O revealed itraconazole and transporter peaks.

The methyl ester provided yields of 70% and greater, while yields obtained using the Br-propionic acid/ester pair were 40-50%. The acyl derivative is then reacted with the amine of the delivery-enhancing transporter to form the conjugate. Alternatively, the halogenated acyl group can first be attached to the transporter molecule through an amide linkage, after which the reaction with the drug compound is conducted.

Example 17

Preparation of FK506 Conjugates

This Example describes the preparation of conjugates in which FK506 is attached to a delivery-enhancing transporter. Two different linkers were used, each of which

Linker 2: 2-(2-pyridinyldithio) ethyl hydrazine carboxylate (Scheme III and IV)

A solution of FK506 (1) (0.1g, 124.4μmol), 2-(2-pyridinyldithio) ethyl hydrazine carboxylate (**9**) (0.091g, 373.2μmol) and trifluoroacetic acid (catalytic, 1μL) in anhydrous methanol (5mL) was stirred at room temperature for 16 h. The reaction was monitored by thin layer chromatography that showed almost complete disappearance of the starting material. [TLC solvent system – ethyl acetate $R_f = 0.5$]. The reaction mixture was concentrated to dryness and dissolved in ethyl acetate (20mL). The organic layer was washed with water and 10% sodium bicarbonate solution and then dried over sodium sulfate, filtered and concentrated. The residue was purified by column chromatography using dichloromethane (97): methanol (3) as eluent to give the hydrazone **10** (0.091g, 71%)

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference for all purposes.

9. The method of claim 8, wherein at least one arginine is a D-arginine.

10. The method of claim 9, wherein all of the arginines are D-arginines.

11. The method of claim 7, wherein at least 70 percent of the amino acids that comprise the delivery-enhancing transporter are arginines or arginine analogs.

12. The method of claim 7, wherein the delivery-enhancing transporter comprises at least 5 contiguous arginines or arginine analogs.

13. The method of claim 1, wherein the compound is attached to the delivery enhancing transporter by a linker.

14. The method of claim 13, wherein the linker is a releasable linker which releases the compound from the delivery-enhancing transporter after the compound has passed into and through one or more layers of an epithelial or endothelial tissue.

15. The method of claim 14, wherein the compound is biologically active upon release from the linker.

16. The method of claim 1, wherein the compound is substantially inactive when conjugated to the delivery-enhancing transporter.

17. The method of claim 14, wherein the half-life of the conjugate is between 5 minutes and 24 hours upon contact with the epithelial or endothelial tissue.

18. The method of claim 17, wherein half-life of the conjugate is between 30 minutes and 2 hours upon contact with the epithelial or endothelial tissue.

19. The method of claim 14, wherein the compound is released from the linker by solvent-mediated cleavage.

20. The method of claim 13, wherein the conjugate is substantially stable at acidic pH but the compound is substantially released from the delivery-enhancing transporter at physiological pH.

22. The method of claim **21**, wherein X is selected from the group consisting of N, O, S, and CR_7R_8, wherein R_7 and R_8 are each independently selected from the group consisting of H and alkyl.

23. The method of claim **21**, wherein the conjugate comprises structure 3 and R_2 is selected to obtain a conjugate half-life of between 5 minutes and 24 hours.

24. The method of claim **23**, wherein R_2 is selected to obtain a conjugate half-life of between 5 minutes and 24 hours.

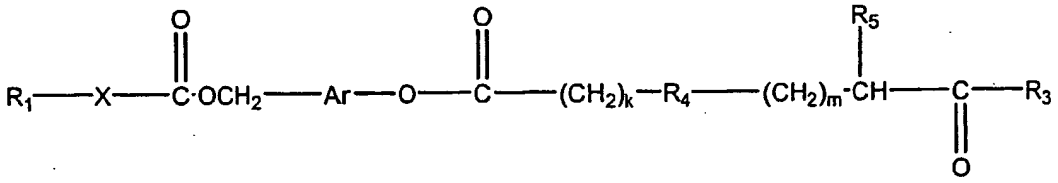
25. The method of claim **21**, wherein the conjugate comprises structure 3, Y is N, and R_2 is methyl, ethyl, propyl, butyl, allyl, benzyl or phenyl.

26. The method of claim **25**, wherein R_2 is benzyl; k, m, and n are each 1, and X is O.

27. The method of claim **21**, wherein the conjugate comprises structure 4; R_4 is S; R_5 is NHR_6; and R_6 is hydrogen, methyl, allyl, butyl or phenyl.

28. The method of claim **21**, wherein the conjugate comprises structure 4; R_5 is NHR_6; R_6 is hydrogen, methyl, allyl, butyl or phenyl; and k and m are each 1.

29. The method of claim **20**, wherein the conjugate comprises structure 6 as follows:



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wherein:

R_1-X comprises the compound;

X is a functional group on the compound to which the linker is attached;

Ar is an aryl group having the attached radicals arranged in an *ortho* or *para* configuration, which aryl group can be substituted or unsubstituted;

38. The method of claim 1, wherein the compound is a diagnostic imaging or contrast agent

39. The method of claim 1, wherein the compound is a non-nucleic acid.

40. The method of claim 1, wherein the compound is a non-polypeptide.

41. The method of claim 1, wherein the compound exerts its biological effect while or after passing into both the epidermis and the dermis.

42. The method of claim 41, wherein the compound acts upon immune cells present in the dermis.

43. The method of claim 1, wherein the compound is a therapeutic for skin disorders or a cosmetic.

44. The method of claim 43, wherein the compound is selected from the group consisting of antibacterials, antifungals, antivirals, antiproliferatives, immunosuppressives, vitamins, analgesics, and hormones.

45. The method of claim 1, wherein the epithelial tissue comprises a blood vessel and the compound enters the blood vessel from the epithelial tissue.

46. The method of claim 45, wherein the compound exerts its biological effect after entry into the capillary system.

47. The method of claim 45, wherein the compound is a systemically active agent.

48. The method of claim 1, wherein the compound is selected from the group consisting of antibacterials, antifungals, antivirals, antiproliferatives, hormones, antiinflammatories, vitamins, and analgesics.

49. The method of claim 48, wherein the compound is an antiinflammatory agent.

61. The method of claim 57, wherein the conjugate is administered topically and the compound is taken up by cells that comprise the follicular or interfollicular epidermis.

62. The method of claim 57, wherein the conjugate is administered by a transdermal patch.

63. The method of claim 57, wherein the conjugate is administered topically and the compound crosses into and across one or both of the papillary dermis and the reticular dermis.

64. The method of claim 63, wherein the compound is taken up by cells present in the dermis.

65. The method of claim 64, wherein the compound is taken up by one or more cells selected from the group consisting of fibroblasts, epithelial cells and immune cells.

66. The method of claim 1, wherein the epithelial tissue comprises a mucous membrane.

67. The method of claim 66, wherein the conjugate is administered by an oral, nasal, pulmonary, buccal, rectal, transdermal, vaginal or ocular route.

68. The method of claim 66, wherein the compound is a systemically active agent.

69. The method of claim 68, wherein the compound is selected from the group consisting of antibacterials, antifungals, antivirals, antiproliferatives, hormones, antiinflammatories, vitamins, and analgesics.

70. The method of claim 1, wherein the epithelial tissue is gastrointestinal epithelium.

80. The method of claim 79, wherein the compound is a therapeutic for treating ischemia, Parkinson's disease, schizophrenia, cancer, acquired immune deficiency syndrome (AIDS), infections of the central nervous system, epilepsy, multiple sclerosis, neurodegenerative disease, trauma, depression, Alzheimer's disease, migraine, pain, and a seizure disorder.

81. A conjugate that comprises a) a compound to be delivered into and across one or more layers of an animal epithelial or endothelial tissue, and b) a delivery-enhancing transporter that comprises 5 to 25 arginine residues; and c) a releasable linker which releases the compound, in biologically active form, from the delivery-enhancing transporter after the glucocorticoid or ascomycin has passed into and across one or more layers of the epithelial or endothelial tissue.

82. The conjugate of claim 81, wherein the delivery-enhancing transporter comprises 7 to 15 arginine residues or arginine analogs.

83. The conjugate of claim 81, wherein the delivery-enhancing transporter consists essentially of 5 to 50 amino acids, at least 50 percent of which amino acids are arginines or arginine analogs.

84. The conjugate of claim 81, wherein the delivery-enhancing transporter comprises at least 5 contiguous arginines or arginine analogs.

85. The conjugate of claim 81, wherein the half-life of the conjugate is between 5 minutes and 24 hours upon contact with the epithelial or endothelial membrane.

86. The conjugate of claim 81, wherein the compound is released from the linker by solvent-mediated cleavage.

87. The conjugate of claim 81, wherein the conjugate is substantially stable at acidic pH but the compound is substantially released from the delivery-enhancing transporter at physiological pH.

89. The conjugate of claim 88, wherein X is selected from the group consisting of N, O, S, and CR_7R_8, wherein R_7 and R_8 are each independently selected from the group consisting of H and alkyl.

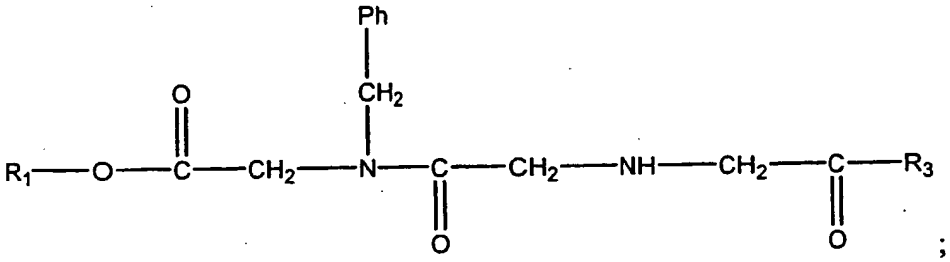
90. The conjugate of claim 88 wherein the conjugate comprises structure 3, Y is N, and R_2 is methyl, ethyl, propyl, butyl, allyl, benzyl or phenyl.

91. The conjugate of claim 90, wherein R_2 is phenyl; k, m, and n are each 1, and X is O.

92. The conjugate of claim 88, wherein the linker comprises structure 4; R_4 is S; R_5 is NHR_6; and R_6 is hydrogen, methyl, allyl, butyl or phenyl.

93. The conjugate of claim 88, wherein the conjugate comprises structure 4; R_5 is NHR_6; R_6 is hydrogen, methyl, allyl, butyl or phenyl; and k and m are each 1.

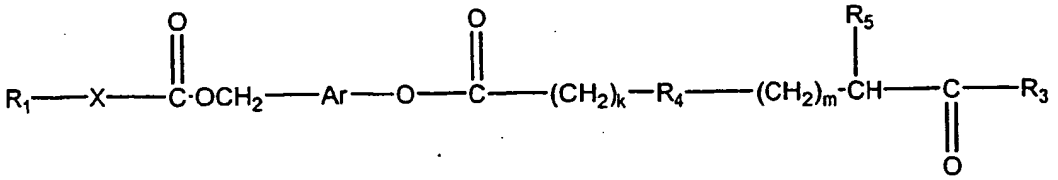
94. The conjugate of claim 88, wherein the conjugate comprises:



;

wherein Ph is phenyl.

95. The conjugate of claim 86, wherein the conjugate comprises structure 6 as follows:



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wherein:

R_1-X comprises the compound;

101. The formulation of claim **100**, wherein the topical dosage form is a transdermal patch.

102. A method for treating a skin inflammatory condition, the method comprising contacting skin affected by the inflammatory condition with a conjugate that comprises a) a glucocorticoid or an ascomycin, and b) a delivery-enhancing transporter that comprises 5 to 25 arginine residues.

103. The method of claim **102**, wherein the inflammatory condition is selected from the group consisting of psoriasis, eczema and alopecia areata.

104. The method of claim **102**, wherein the glucocorticoid is hydrocortisone.

105. The method of claim **102**, wherein the ascomycin is a cyclosporin or FK506.

106. The method of claim **102**, wherein the ascomycin is a cyclosporin.

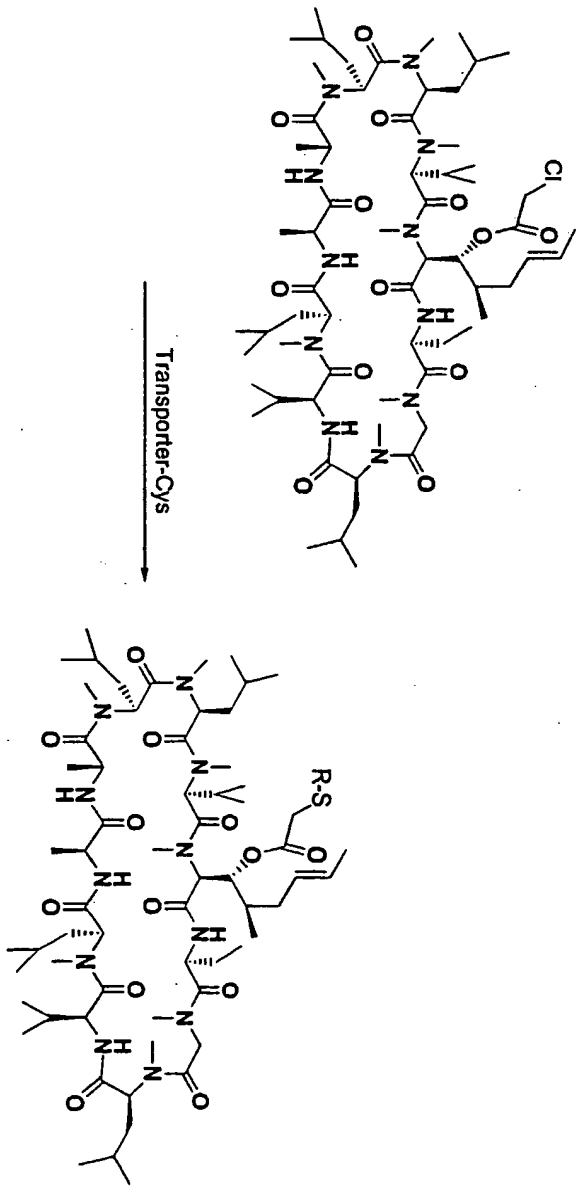


Figure 2

Figure 4

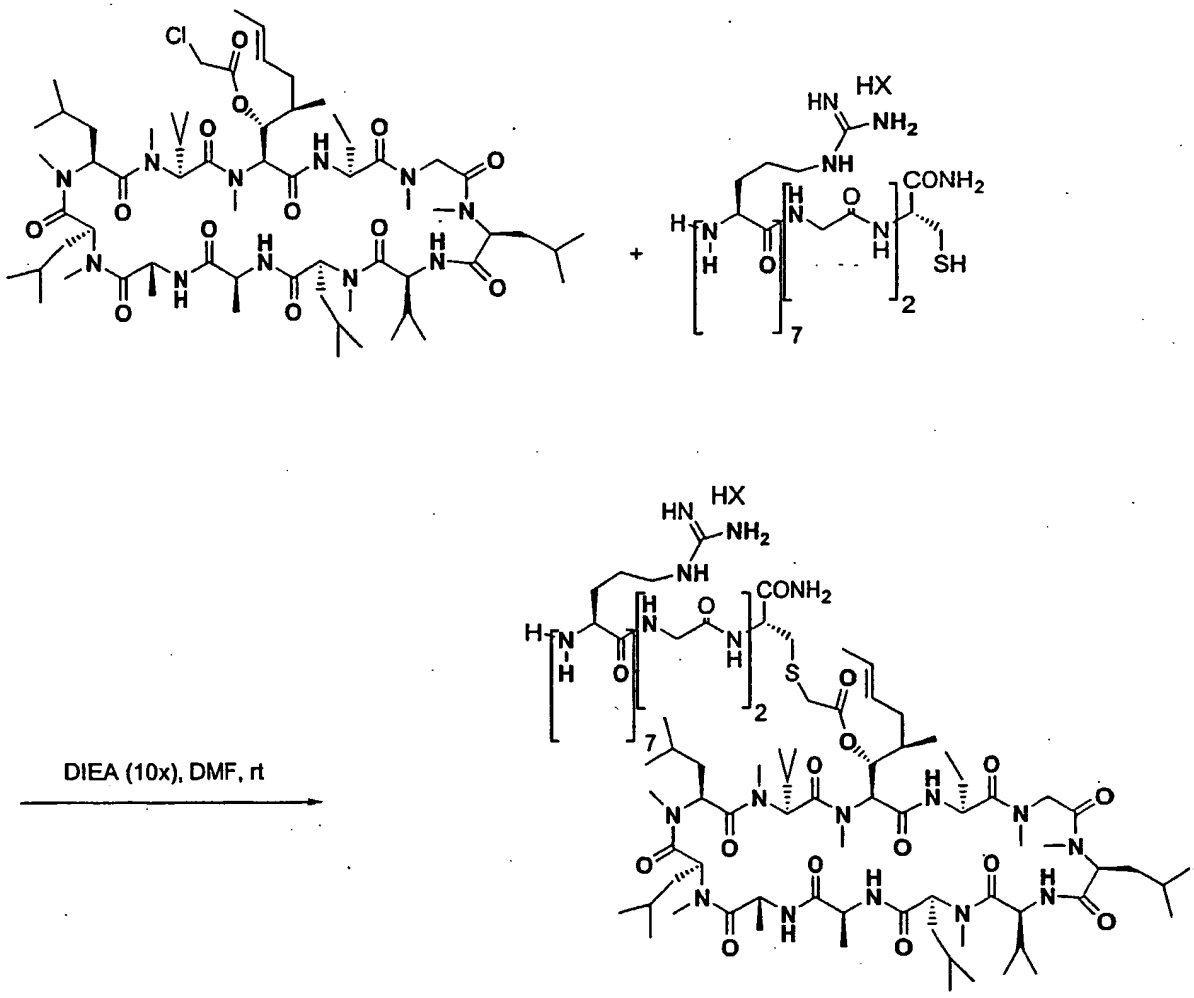
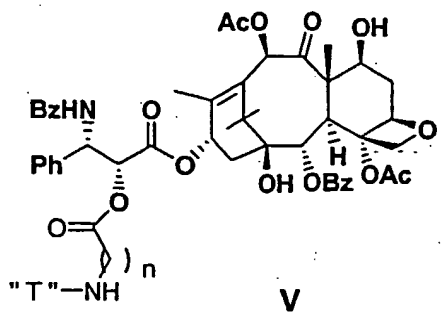


Figure 5E



V

Figure 5F

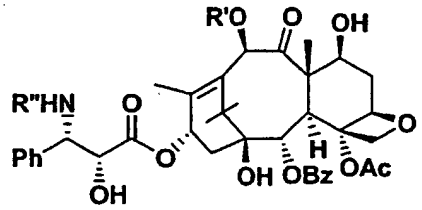


Figure 5G

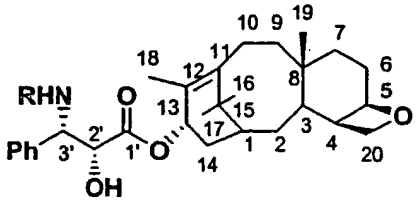


Figure 5H

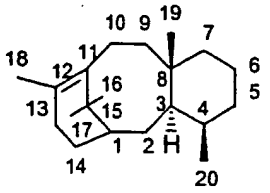


Figure 7

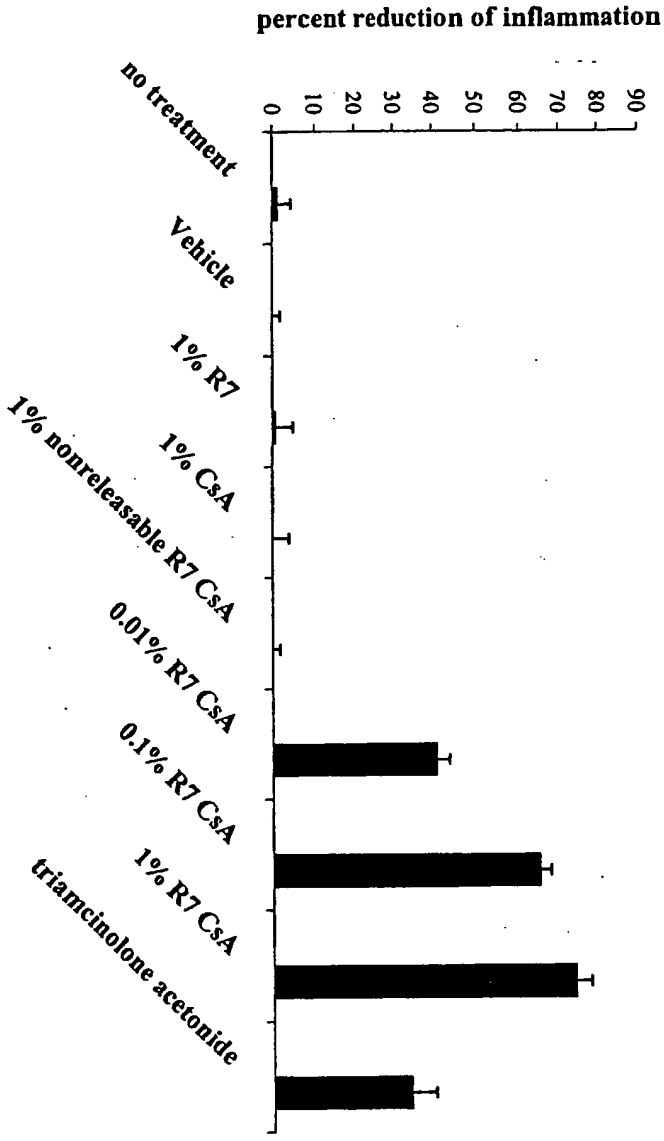

percent reduction of inflammation
0
10
20
30
40
50
60
70
80
90
no treatment
Vehicle
1% R7
1% CsA
1% nonreleasable R7 CsA
0.01% R7 CsA
0.1% R7 CsA
1% R7 CsA
triamcinolone acetonide

Figure 10

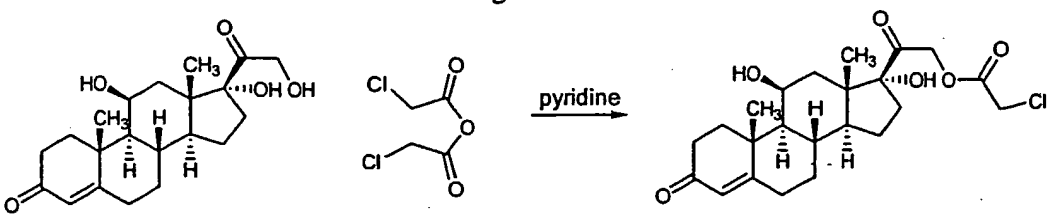


Figure 11

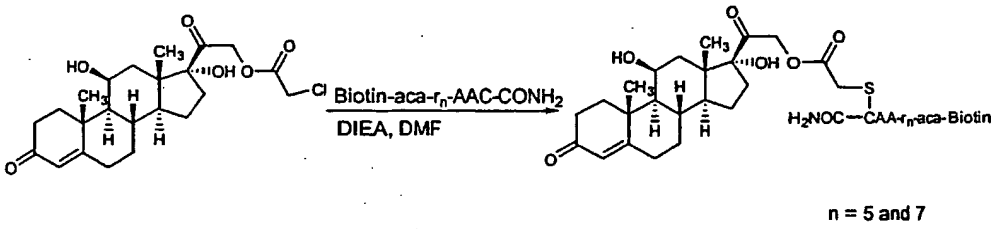


Figure 14

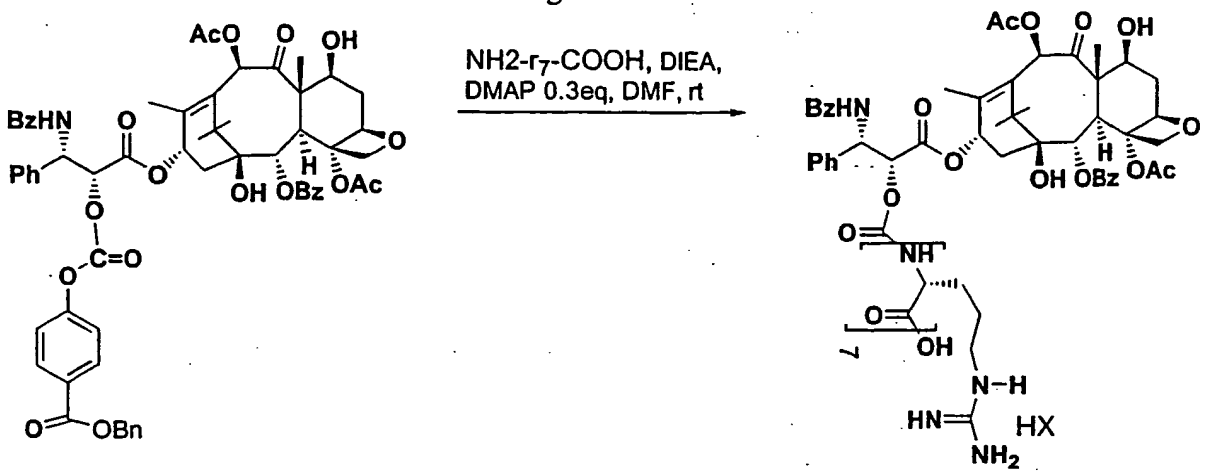

NH2-r7-COOH, DIEA,
DMAP 0.3eq, DMF, rt
AcO
O
OH
BzHN
Ph
OBz
OAc
C=O
OBn
NH
N-H
HN
HX
NH2

Figure 16

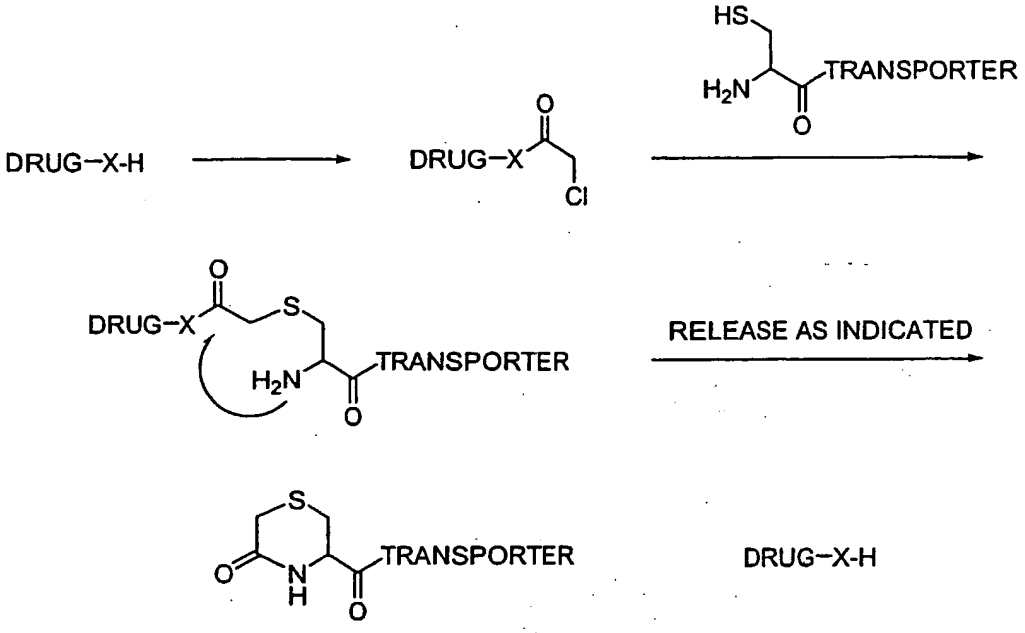


Figure 17

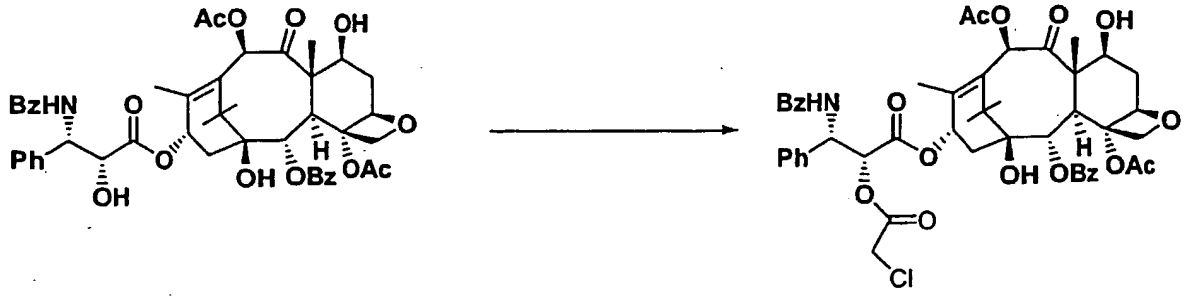


Figure 19

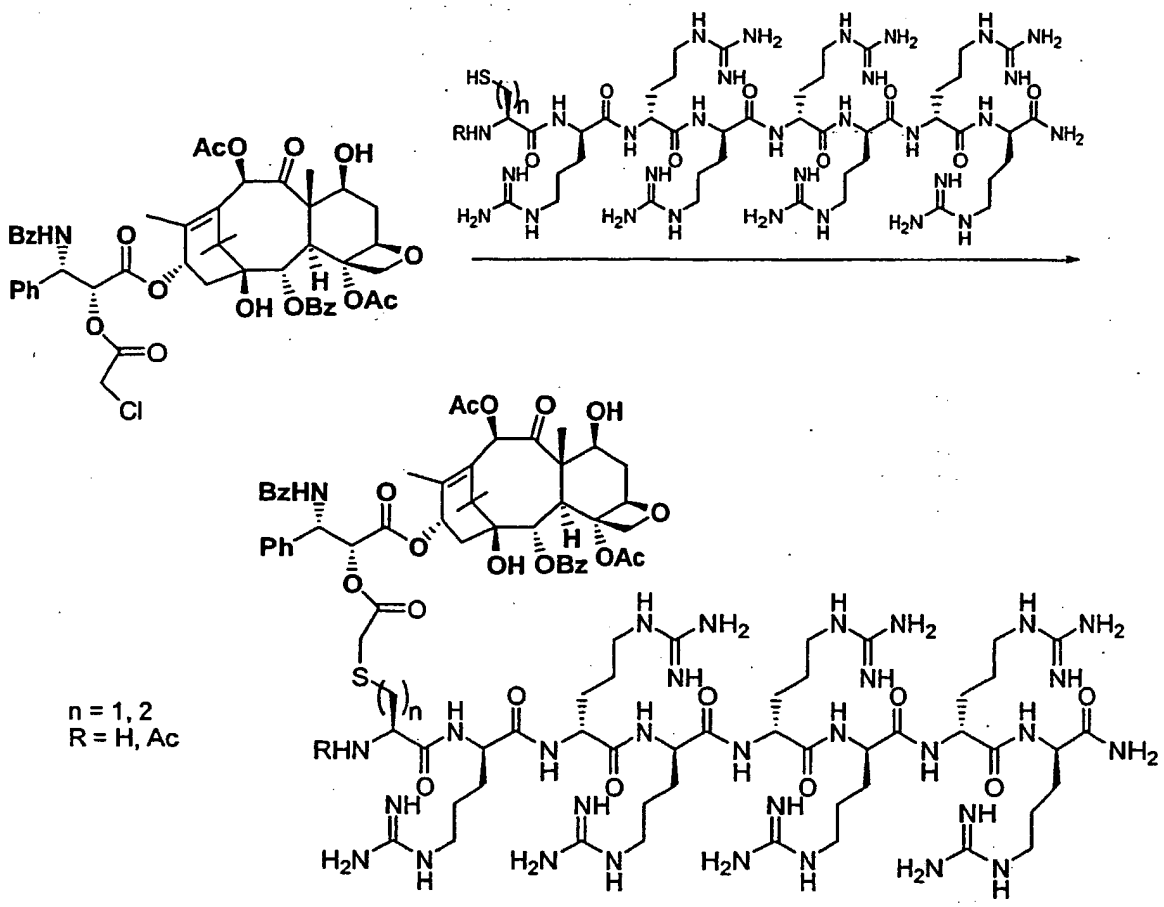


Figure 21

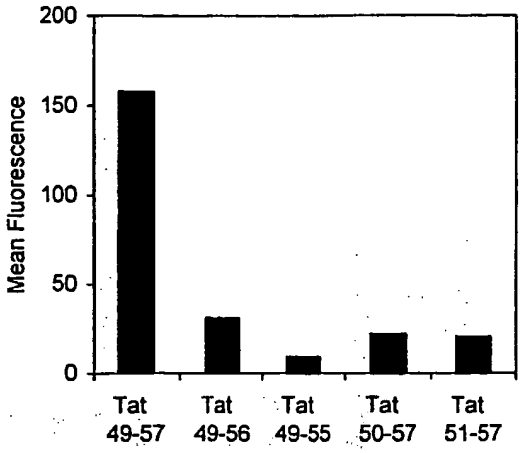

200
150
100
50
0
Mean Fluorescence
Tat 49-57
Tat 49-56
Tat 49-55
Tat 50-57
Tat 51-57

Figure 23

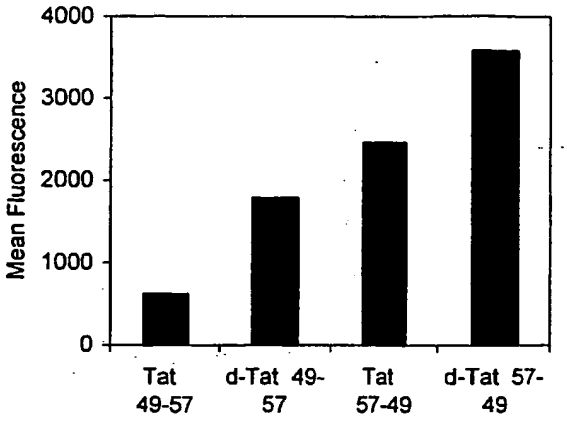

4000
3000
2000
1000
0
Mean Fluorescence
Tat 49-57
d-Tat 49-57
Tat 57-49
d-Tat 57-49

Figure 25

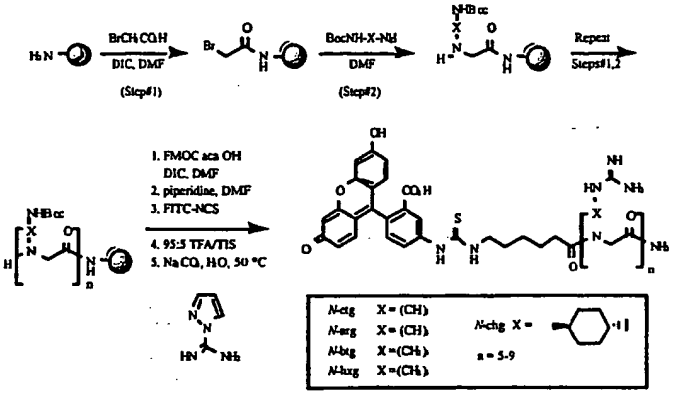

BrCH2CO2H
DIC, DMF
(Step#1)
BocNH-X-NH
DMF
(Step#2)
Repeat
Steps#1,2
1. FMOC aca OH
DIC, DMF
2. piperidine, DMF
3. FITC-NCS
4. 95:5 TFA/TIS
5. Na2CO3, H2O, 50 °C
n-atg X = (CH2)
n-arg X = (CH2)
n-hig X = (CH2)
n-lysg X = (CH2)
n-chg X =
n = 5-9

Figure 27

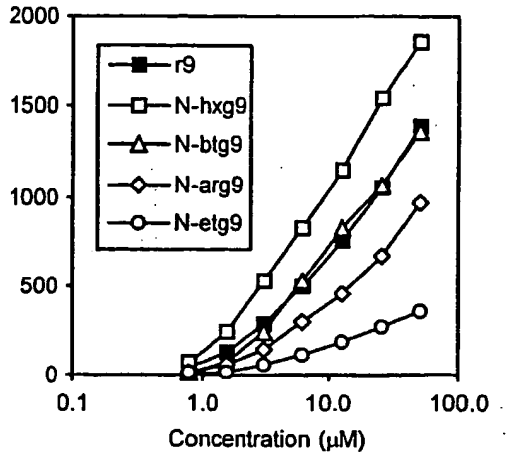

2000
1500
1000
500
0
0.1
1.0
10.0
100.0
Concentration (μM)
r9
N-hxg9
N-btg9
N-arg9
N-etg9

Figure 29

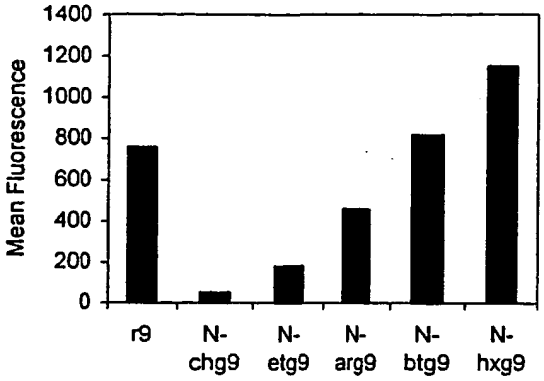
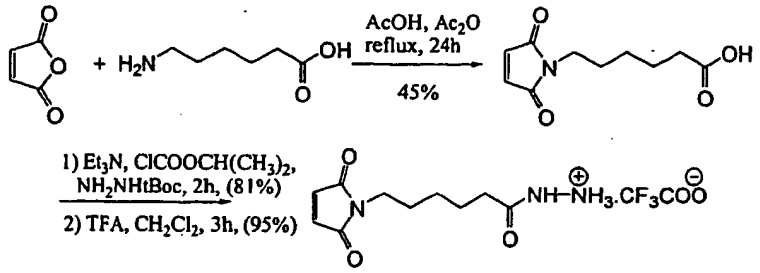

Mean Fluorescence
1400
1200
1000
800
600
400
200
0
r9
N-chg9
N-etg9
N-arg9
N-btg9
N-hxg9

Figure 31A

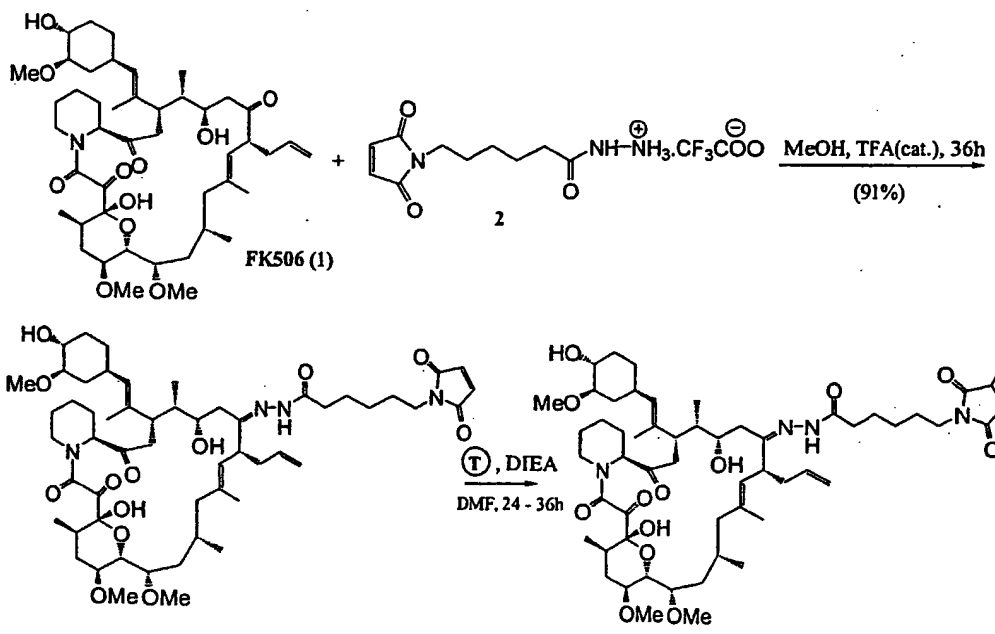
Synthetic Schemes for FK 506 Conjugates

Scheme I (*Linker: 6-Maleimido caproic hydrazide, TFA salt)*



Ref. Willner et al; Bioconjugate Chemistry, 1993, 4, 521-527

Scheme II



T = (4) Bacar$_9$C = CGC-1004 (73%)
(5) Bacar$_7$C = CGC-1005 (50%)
(6) BacaC = CGC-1006 (52.9%)
(7) r$_7$C = CGC-1007 (43.8%)
(8) R$_7$C = CGC-1022 (62.8%)

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(72) Inventors: **ROTHBARD, Jonathan, B.**; 19500 Prunerridge Avenue, Apt.#1203, Cupertino, CA 95014 (US). **WENDER, Paul, A.**; 930 Siskiyou Avenue, Menlo Park, CA 94025 (US). **McGRANE, P., Leo**; 110 Beacon Street, Mountain View, CA 94040 (US). **SISTA, Lalitha, V.**, S.; 1333 Floyd Avenue, Sunnyvale, CA 94087 (US). **KIRSCHBERG, Thorsten, A.**; 360 Chiquita Avenue #11, Mountain View, CA 94041 (US).

(74) Agents: **SMITH, Timothy, L.** et al.; Townsend and Townsend and Crew LLP, 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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WO 01/13957 A3

(54) Title: ENHANCING DRUG DELIVERY ACROSS AND INTO EPITHELIAL TISSUES USING OLIGO ARGININE MOIETIES

(57) Abstract: This invention provides compositions and methods for enhancing delivery of drugs and other agents across epithelial tissues, including the skin, gastrointestinal tract, pulmonary epithelium, and the like. The compositions and methods are also useful for delivery across endothelial tissues, including the blood brain barrier. The compositions and methods employ a delivery enhancing transport that has sufficient guanidino or amidino sidechain moieties to enhance delivery of a compound conjugated to the reagent across one or more layers of the tissue, compared to the non-conjugated compound. The delivery enhancing polymers include, for example, poly-arginine molecules that are preferably between about 6 and 25 residues in length.

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 92 07871 A (ALLELIX BIOPHARMA) 14 May 1992 (1992-05-14) examples ---	3,38
X	CHEMICAL ABSTRACTS, vol. 123, no. 7, 1995 Columbus, Ohio, US; abstract no. 79357, SUMNER-SMITH, M. ET AL: "Antiherpetic activities of N-.alpha.-acetyl-nona-D-arginine amide acetate" XP002086015 abstract & DRUGS EXP. CLIN. RES., 1995, VOL. 21, NO. 1, PAGE(S) 1-6, ---	3,38
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